Haematopoietic progenitor cells in carotid disease

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HAEMATOPOIETIC PROGENITOR CELLS IN CAROTID DISEASE

A thesis presented by

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for the degree of MD (Res) in the School of Medicine,

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June 2012

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Statement of Originality

The work described in this thesis was carried out in the Academic Department of Surgery, Cardiovascular Division, St Thomas' Hospital, King's College London between 2005 and 2008. It is the original work of the author, who carried out all the experiments under the guidance of his supervisors and with the assistance of colleagues within the department. This work has not previously been submitted for a degree or any other qualification at any other university.

Acknowledgements

My greatest thanks go to Professor Alberto Smith and Professor Kevin Burnand, firstly for giving me the job in the first place, but most importantly for their tireless efforts and the constant stream of ideas and support that have made this thesis possible. I would also like to thank Matthew Waltham and Julia Humphries and all the other members of the Academic Department of Surgery for all your time and effort over the past few years. I genuinely feel very lucky to have known you all and will always look back at my time at St Thomas' with great pride.

Special thanks go to the British Heart Foundation for awarding me a Fellowship through which they largely funded this work and to the Society of Academic and Research Surgery for awarding me a bursary.

None of this would have been possible without the constant love and support of my family and in particular my wife. My parents have also been wonderful and have stepped in to take the children on many occasions. My wife and two children have been and continue to be the greatest source of happiness in my life and I now hope to make up for some lost time with them.

Grants and prizes supporting this work

Society of Academic and Research Surgery – Academic Bursary	2007
British Heart Foundation Clinical Fellowship	2005

Presentations arising from this work

Patel SD, Wadoodi A, Waltham M, Ahmad A, Burnand KG, Smith A. Circulating Progenitor cell function and response after carotid endarterectomy protects against restenosis. Society of Academic and Research Surgery (SARS) Bristol 2009. *Patey Prize Session*

Patel SD, Wadoodi A, Waltham M, Burnand KG, Smith A. Increased endothelial progenitor cell (EPC) number and function is associated with symptomatic carotid artery disease. SARS Birmingham 2008. *Patey Prize Session*

Patel SD, Wadoodi A, Waltham M, Burnand K, Smith A. False positives in the detection of circulating endothelial progenitor cells by flow cytometry.
SARS Cambridge 2007

Publications arising from this work

Patel SD, Humphries J, Mattock K, Wadoodi A, Modarai B, Ahmad A, Burnand KG, Waltham M, Smith A.Haematopoietic Progenitor Cells and Restenosis After Carotid Endarterectomy. Stroke. 2012 Apr 17. [Epub ahead of print]

Patel SD, Waltham M, Wadoodi A, Burnand KG, Smith A. The role of endothelial cells and their progenitors in intimal hyperplasia. Ther Adv Cardiovasc Dis. 2010 Apr;4(2):129-41

Abstract

Introduction

Haematopoietic progenitor cells (HPCs) may attenuate the response to acute vascular injury by maintaining endothelial integrity and function. The aim of this study was to determine whether circulating HPC number and function, and mobilising cytokines, reflect significant carotid disease or correlate with restenosis following carotid endarterectomy (CEA).

Methods

Initially the assay conditions to measure HPC number and function were optimized. HPC numbers were measured by flow cytometry ($CD133^{+ve}/CD34^{+ve}$) and early colony forming unit assay (eCFU). HPC function was measured by migration assay and by staining for senescence associated B-galactosidase (SA-Bgal). HPC number and function was then measured in 62 patients undergoing CEA pre-operatively, 1 day post operatively and 6 weeks post-operatively. Restenosis was assessed by duplex scanning at 3, 6 and 12 months. The circulating profile of GM-CSF, PlGF, SDF1 and VEGF was measured by multiplex ELISA.

Results

HPC numbers ($P<0.001$) and eCFU counts ($P=0.001$) fell rapidly 24hrs after CEA. The percentage post-operative fall in $CD133^{+ve}/CD34^{+ve}$ cell numbers negatively correlated with degree of restenosis at the 6 month scan ($r= -0.38$, $p=0.013$). The percentage fall in eCFU number negatively correlated with degree of restenosis at

the 6 (R=-0.42, P=0.008) and 12 month scans(R=-0.49, P=0.026). The migration rate of HPCs isolated from pre-operative blood also negatively correlated with restenosis at the 6 (R=-0.5, P=0.009) and 12 month scans(R=-0.53, P=0.05). Pre-operative SDF1 levels correlated with falls in $CD133^{+ve}/CD34^{+ve}$ number (R=0.42, P=0.044) and eCFU counts (R=0.56, P=0.004), though not with restenosis.

Conclusion

HPC function appears to be linked to the development of carotid artery restenosis following endarterectomy. These data support the concept that HPCs have a role in regulating remodelling of the unstable and injured arterial wall.

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Abbreviations

CCAPAV	Common carotid artery peak systolic velocity
CCR-2	Chemokine receptor-2
CEA	Carotid endarterectomy
EC	Endothelial cell
ECM	Extracellular matrix
EPC	Endothelial progenitor cell
EPC-CFU	Endothelial progenitor cell colony forming unit
DAPI	4',6-diamidino-2-phenylindole
Dil- acLDL	1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate – acetylated low density lipoprotein
DMEM	Dulbecco's modified eagle medium
DPBS	Dulbecco's phosphate buffered saline
EBM	Endothelial basement medium
EGM-2	Endothelial growth medium-2
FCS	Fetal calf serum
FGF-1	Fibroblast growth factor-1
FITC	Fluorescein isothiocyanate
FSC	Forward Scatter
GM-CSF	Granulocyte-macrophage colony stimulating factor
ICAPSV	Internal carotid artery peak systolic velocity
MCP-1	Monocyte chemoattractant protein -1
MSC	Mesenchymal stem cell
PBS	Phosphate buffered saline
PBMNC	Peripheral blood mononuclear cell
PE	phycoerythrin

PerCP	Peridinin chlorophyll protein
PDGF	Platelet derived growth factor
PlGF	Placental derived growth factor
SA-Bgal	Senescence associated B-galactosidase
SDF-1	Stromal derived factor -1
SMC	Smooth muscle cell
SSC	Side Scatter
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
UEA-1	Ulex Europaeus Agglutinin

Introduction

1.1 Carotid artery stenosis

Stroke is the third most common leading cause of death in the UK after ischaemic heart disease and cancer and is the principal cause of neurological disability. The annual incidence of stroke is 2 per 1000 and 125 000 patients will suffer their first stroke each year[1]. The annual incidence of transient ischaemic attack (TIA) is 0.5 per 1000 [2]. About 80 % of all strokes are ischaemic and the remainder haemorrhagic (intracerebral/subarachnoid). Carotid artery atherosclerosis and subsequent thromboembolism of the internal carotid artery (ICA) is the most common cause of ischaemic stroke and transient ischaemic attacks [3] (Fig1). These thromboembolic events are related to plaque instability caused by plaque ulceration, rupture and subsequent thrombosis [4].

1.1.1 Presentation of carotid disease

A patient with carotid artery stenosis is considered symptomatic if the patient has transient or permanent focal neurologic symptoms related to the ipsilateral retina or the cerebral hemisphere. Symptoms of carotid artery stenosis include ipsilateral transient visual obscuration (amaurosis fugax) from retinal ischaemia; contralateral weakness or numbness of an arm, a leg, or the face, or a combination of these sites; visual field defect; dysarthria; and, in the case of dominant (usually left) hemisphere involvement, aphasia.

In daily clinical practice, carotid artery stenosis is found in many patients during evaluation of ill-defined episodes of “dizziness,” generalized subjective weakness, syncope or near-syncope episodes, “blurry vision,” transient positive visual phenomena (such as “floaters” or “stars”) or in the investigation of carotid bruit found on general medical examination. These nonspecific symptoms in patients with carotid artery stenosis do not qualify as symptomatic ischemic events; these patients are considered asymptomatic even in the presence of high-grade carotid artery stenosis.

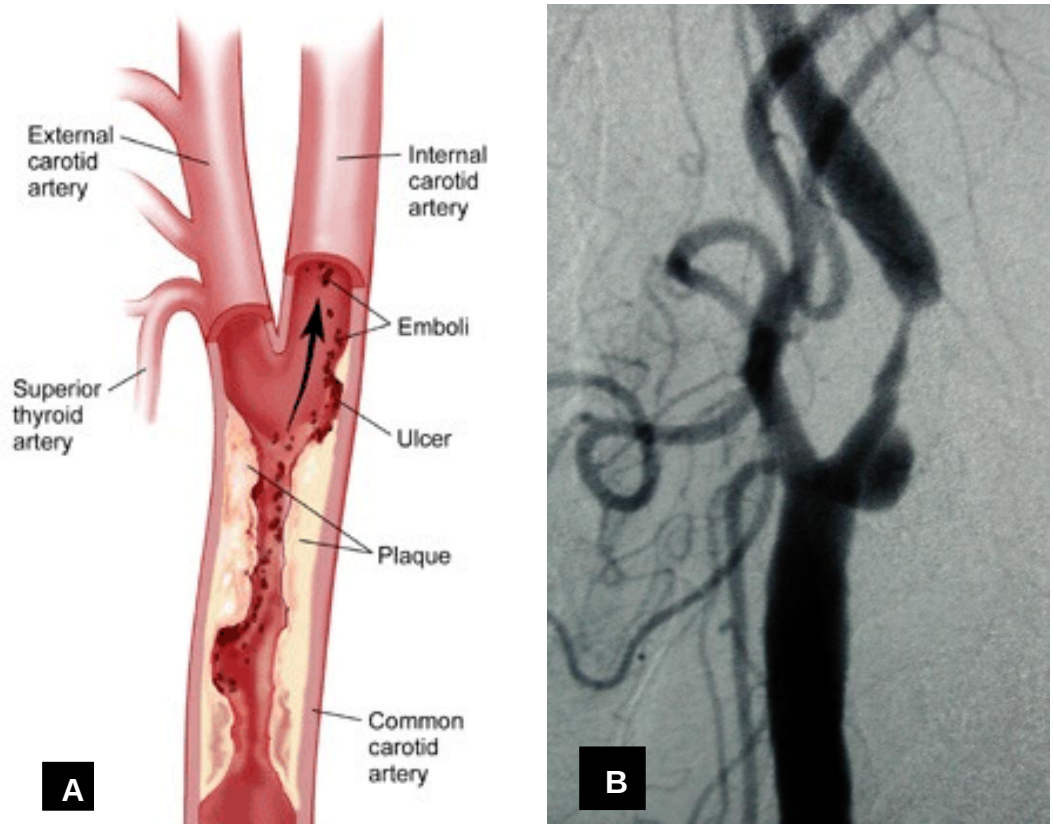


Fig 1. Carotid artery stenosis.

A. Anatomy of the bifurcation of the common carotid artery (CCA). Emboli generated at the plaque surface travel up the internal carotid artery (ICA) causing cerebrovascular symptoms[3, 4]. B. An angiogram showing an ICA stenosis.

1.1.2 Investigation of carotid stenosis

Doppler ultrasonography is the main diagnostic imaging tool used to screen for carotid artery stenosis. The advantages of doppler include its low cost, non-invasive nature and accessibility. It is, however, highly dependent on operator experience and skill. Doppler ultrasonography has a sensitivity of 86% and a specificity of 87% for the detection of haemodynamically significant carotid artery stenosis when compared with catheter angiography [5].

Catheter angiography is still regarded as the standard for defining the degree of stenosis and the morphologic features of the plaque, but this technique is neither feasible nor recommended in day to day practice because of its risks and costs. Computed tomographic angiography (CTA) and magnetic resonance angiography (MRA) have gained increasing popularity for use in the diagnosis of carotid artery stenosis, replacing conventional catheter angiography. MRA has a sensitivity of 95 % and a specificity of 90% when compared with angiography for the detection of 70-99% stenosis [5]. CTA is a minimally invasive technique which allows significant stenosis to be detected not only in the cervical portion of the carotid artery but also in the cranial portion and down to the aortic arch. In a recent systematic review on the performance of non-invasive imaging modalities in the assessment of a 70-99% stenosis, contrast enhanced MRA was the most accurate imaging modality (specificity 94%, sensitivity 93%) followed by CTA (94% and 76% respectively) and duplex (84% and 89% respectively) [6]. Though practice varies between centres, MRA or CTA may be used as confirmatory tests after results of a Doppler study are suggestive of haemodynamically significant stenosis.

1.1.3 Management of carotid disease

1.1.3.1 Best medical management

All patients with cerebrovascular disease benefit from optimisation of risk factors and exclusion of comorbidities, whether they are to undergo surgery or not. An ECG should be performed to look for occult cardiac pathology and bloods test performed to look for evidence of diabetes, arteritis, polycythaemia, thrombocytosis, sickle cell disease and hyperlipidaemia. The European stroke initiative [7] defined what constitiutes 'best medical therapy'. Angina therapy must be optimised because the principal cause of late death is cardiac. Blood pressure should be maintained below 140/90 or 130/80 in diabetics. All patients (symptomatic and asymptomatic) should be treated with a statin unless contraindicated as established in the Heart Protection Study[8]. Treatment with statins cause on average a 25% relative risk reduction in: (i) any major coronary event, (ii) any stoke and (iii) the need for revascularisation at 5 years. This benefit is irrespective of age, gender or presenting cholesterol level. Patients should also be treated using antiplatelet therapy unless contraindicated. Aspirin remains the first line agent and meta-analysis suggests that it reduces the long term risk of stroke by 25%[9]. Other recommendations include optimising glycaemic control, stopping smoking, reducing alcohol, salt and fat intake, regular physical exercise and weight loss if BMI is elevated. Rapid institution of 'best medical therapy' after the presenting stroke/TIA leads to a significant reduction in the 90 day risk of further stroke from 10.3% to 2.1% [10].

1.1.3.2 Surgical management of carotid disease

The role of carotid endarterectomy (CEA) in the management of patients with carotid stenosis has been extensively investigated and defined through large randomised controlled trials (RCTs). The largest of these trials are the North American Symptomatic Carotid Endarterectomy Trial (NASCET)[11,12], the European Carotid Surgery Trial (ECST)[13] and the Veterans Affairs Cooperative Study Group[14]. The Carotid Endarterectomy Trialist Collaboration (CETC)[15, 16] have combined the results of these studies forming a database that includes 5-year outcomes from >6000 patients.

CEA is not indicated in symptomatic patients with a 0-50% stenosis. In recently symptomatic patients (<6 months) with a 50-69% stenosis, CEA confers a significant benefit with a 5-year stroke risk of 28% with medical management alone compared with 20% when combined with surgery (relative risk reduction 28%, numbers needed to treat to prevent 1 stroke at 5 years is 13). CEA confers maximum benefit in patients who are recently symptomatic and have a 70-99% stenosis, with a 5-year stroke risk of 33% with medical management alone compared with 17% when combined with surgery (relative risk reduction 48%, numbers needed to treat to prevent 1 stroke at 5 years is 6). CEA does not confer any long term benefit in patients with near-occlusion.

The decision on whether to implement invasive treatment in patients with asymptomatic carotid artery stenosis has been informed by two large RCTs

comparing CEA with medical treatment for asymptomatic patients[17,18]. These showed that surgery provides only a modest benefit in stroke prevention, with CEA reducing the risk of stroke from 2% per year to 1% per year. In the Asymptomatic Carotid Surgery Trial[18], surgery did not benefit patients aged 75-years or older because of the excess mortality rate at follow-up in these patients (a rate related to non cerebrovascular events such as myocardial infarction and cancer). These trials have been criticized because the medical treatment arm was not uniformly defined and did not include interventions currently considered optimal medical management, such as aggressive reduction in blood pressure and lipid concentrations. It is unknown whether current standard medical therapy can decrease the relative benefit from CEA in patients with asymptomatic carotid artery stenosis by further decreasing the rate of stroke.

1.2 Restenosis

Recurrent arterial narrowing (restenosis) is the major complication limiting the success of revascularisation procedures. It can occur after any cardiovascular intervention including coronary and peripheral angioplasty[19], bypass grafting[20], endarterectomy[21], and arterio-venous fistula formation[22]. Restenosis is caused by a combination of processes. In the short term, it may be caused by elastic recoil of the vessel wall, thrombus formation at the site of injury, and variations in operative technique that lead to a smaller anastomosis or kinking of the vessel. Longer term patency over the proceeding months to years is limited by intimal hyperplasia,

involving the proliferation and migration of intimal smooth muscle cells[23,24]. Preventing intimal hyperplasia is an important therapeutic target and strategies include not only continued development of stent design and coating materials[25,26], but also manipulation of the cellular response to vascular injury.

1.2.1 The clinical impact of restenosis

The overall incidence of restenosis is approximately 30% a year after coronary angioplasty and bare metal stenting [27] and there is a similar incidence following angioplasty for peripheral arterial disease [28]. The risk of this complication is less after carotid endarterectomy (10%-20%) [29-31]. Restenosis in the coronary artery can lead to severe morbidity and mortality and frequently requires repeated treatment. A study of over 3000 patients who had undergone coronary angioplasty were evaluated between 4-months to 1-year after the procedure[32]. Restenosis occurred in around 50% of patients and significantly more patients with restenosis than without restenosis had angina (70.7% vs 38.7%). After 6-year follow-up, the survival rate was similar in both groups (94%), however, restenosis was associated with a significantly higher rate of MI, CABG and repeat angioplasty[32]. Although these data suggest that the presence of restenosis does not confer an increase in mortality, a patient's quality of life can be detrimentally affected[33]. In the Optimum Percutaneous Transluminal Coronary Angioplasty Compared with Routine Stent Strategy (OPUS-1) trial, restenosis was associated with more frequent angina, more physical limitations and reduced quality of life scores[33]. Restenosis following

carotid endarterectomy carries a 1-10% risk of stroke[30,31]. The economic impact of repeated revascularisation procedures is also considerable[34].

1.2.2 Risk factors for restenosis

The risk of restenosis is almost twice as high among patients with diabetes mellitus. In a study of 954 patients undergoing PCI, target lesion revascularization was required in 28% of patients with insulin-dependent diabetes compared with 16.3% in individuals without diabetes[35]. The high risk for restenosis among patients with diabetes may be associated with metabolic alterations that promote endothelial dysfunction, accelerate intimal hyperplasia, and increase platelet aggregability and thrombogenicity[36]. There is also evidence to suggest that some patients may be genetically predisposed to restenosis. Genetic abnormalities associated with high risk for restenosis include polymorphisms in genes coding for angiotensin-converting enzyme inhibitor [37], glycoprotein receptor IIIa PLA1/2[38] and haptoglobin 2/2 [39].

The choice of material for bypass grafts has an important impact on the risk of restenosis. The patency rates for femoro-popliteal bypass for chronic lower limb ischaemia at 5 years are 66% for vein (any level), 47% for above knee PTFE and 33% for below knee PTFE[40]. Lesion characteristics that may influence risk for restenosis include disease in saphenous vein grafts, length of the lesion, and minimum lumen diameter before stenting[41]. A larger lumen diameter after angioplasty correlates directly with a reduced rate of restenosis [41].

Table 1. Risk factors for restenosis

Patient factors
Restenosis at another treated site
Diabetes mellitus
Smoking
Intervention in acute coronary syndromes
Genetic predisposition
Lesion characteristics and procedural variables
Diameter of vessel
Length of lesion or stent
Lumen diameter before and after procedure
Ostial lesions
Bypass graft material
Total number of occlusions

Drug-eluting stents (DES) have been developed as a means of preventing intimal hyperplasia following angioplasty. They appear to have reduced the early risk of coronary restenosis, though this still occurs in over 10% of stented vessels at 12 months [27]. DES are not suitable for all patients with coronary artery disease. Certain anatomic and clinical scenarios, such as patients with diabetes mellitus,

restenotic lesions after DES, bypass graft disease, and bifurcations, continue to be problematic for restenosis[42, 43]. DES do not improve long-term prognosis[44] There are also concerns of an increased incidence of stent thrombosis and subsequent myocardial infarction in patients who have had a drug eluting stent inserted[27] which necessitates the continuation of post-stent antiplatelet regimen. These rare, life-threatening complications are, in part, caused by impaired endothelial regeneration (*re-endothelialisation*), which is essential in the normal healing process of injured vessels [45]. Sirolimus and paclitaxel are potent anti-mitotic agents that strongly inhibit smooth muscle proliferation and matrix growth, preventing neointimal formation and restenosis. Delayed vessel wall healing because of impaired re-endothelialisation after DES stenting may go hand-in-hand with neointimal suppression [46]. Recent evidence suggests that DES lead to impaired endothelial function at the site of stenting as well as in the distal segments[47].

It is clear that new strategies are therefore needed to prevent intimal hyperplasia that are applicable to the full spectrum of restenotic disease and that enhances vessel wall repair and endothelial function.

1.3 Mechanisms of intimal hyperplasia

Lumen loss after cardiovascular interventions can be separated into 3 distinct stages: early loss associated with elastic recoil, intermediate loss over several months caused by neointimal hyperplasia and late loss caused by negative remodelling (Fig 2). Animal models and human post-mortem specimens show that the mechanism of intimal hyperplasia is similar to that regulating wound healing[48, 49]. Both processes can be divided into three overlapping phases: an inflammatory phase, a granulation or cellular proliferation phase, and a phase of remodelling of the extracellular matrix (ECM) and protein synthesis.

1.3.1 Elastic recoil

Elastic recoil is a dynamic and progressive phenomenon that occurs immediately following angioplasty and results in an immediate loss in lumen diameter. Up to a 34% loss in lumen diameter can be observed within 15-minutes of balloon inflation[50]. Elastic recoil may ultimately account for up to 50% of the loss in acute lumen gain during angioplasty[51].

1.3.2 Intimal hyperplasia

Inflammatory phase (hours to days): Intimal hyperplasia is initiated by factors which vary according to the procedure, and include vasospasm[52], ischaemia caused by damage to the vaso-vasorum[53] and haemodynamic changes particularly

with vein grafts which are exposed to a greater shear stress when transplanted into the arterial circulation[54]. Immediately following angioplasty, fracture of the 'hard' atherosclerotic plaque exposes the thrombogenic contents of the plaque (proteins such as tissue factor, vWF and collagen) to the flowing blood (Fig 2A). These factors lead to endothelial damage and loss and trigger platelet adhesion, activation and thrombin generation, and occasionally thrombus formation at the site of injury[55] (Fig 2B). Thrombin is a potent mitogen that promotes smooth muscle cell (SMC) proliferation and inhibition of this coagulation factor reduces intimal hyperplasia in an animal model[56].

Activated platelets on the injured vessel surface express adhesion molecules, such as P-selectin, that are released from α-granules. The initial tethering and rolling of leukocytes on platelets are mediated by P-selectin binding to leukocyte receptors, such as P-selectin glycoprotein ligand-1 (PSGL-1) [57]. Leukocytes adhere firmly to the platelets through leukocyte integrin Mac-1 (CD11b/CD18) via platelet glycoprotein Ibα (GPIbα) interaction and through cross-linking with fibrinogen to the GPIIb/IIIa receptor[58] (Fig 2C). Leukocytes migrate across the platelet – fibrin layer and into the vessel wall, driven by chemical gradients of chemotactic cytokines, produced in response to injury by smooth muscle cells (SMCs) and leukocytes resident in the medial layer [49]. Mac-1 is thought to be an important signalling protein in the mechanism of restenosis, as blockade of this adhesive molecule[59] or deletion of the Mac-1 gene[60] results in reduced neointimal thickening after experimental angioplasty and stenting.

Cellular proliferation phase (days to weeks): Under normal conditions, vascular SMCs are quiescent and exhibit low levels of proliferative activity. Vascular injury and the subsequent inflammatory response, trigger SMC proliferation through the G1/S transition of the cell cycle[61, 62]. The different phases of the cell cycle are regulated by a series of protein complexes comprising cyclins, cyclin-dependent kinases (CKDs) and their cyclin-dependent kinase inhibitors (CKIs). CKIs, such as p27Kip1 or p21Cip1, regulate the G1/S transition through binding to cyclins E/CDK2 and inhibiting CDK2 activity, leading to cell cycle arrest. Arterial injury produces down regulation of p27Kip1, which triggers an increase in cell proliferation. Gene transfer of p27Kip1 or p21Cip1 into balloon-injured arteries produces a significant reduction in SMC proliferation and neointimal thickening[62-64]. Deficiency of p27Kip1 results in a prominent vascular phenotype with markedly increased neointimal thickening and inflammatory cell accumulation after mechanical arterial injury[65]. These findings suggest that p27Kip1 and p21Cip1 are endogenous regulators of G1 transit in vascular SMCs and inhibit cell proliferation after arterial injury.

The cellular proliferation phase is marked by the release of growth and chemotactic factors from activated platelets, leukocytes, surrounding endothelial cell (ECs) as well as medial SMCs. These include platelet derived growth factor A and B (PDGF A and B), fibroblast growth factor (FGF), insulin like growth factor (IGF), interleukins(IL) IL-1, IL-6, IL-8 and vascular endothelial growth factor (VEGF)[48, 49].

These stimulate the migration and proliferation of SMCs from the media into the neointima (Fig 2D). They also promote proliferation of surrounding ECs and their migration into the denuded area. This is thought to be the predominant process leading to re-endothelialisation [49, 66]. Extracellular matrix proteoglycans and hyaluronan are synthesised by SMCs and participate in regulation of vascular permeability, lipid metabolism, and thrombosis [67]. The resultant neointima consists of SMCs, extracellular matrix, and macrophages recruited over several weeks (Fig 2E).

Remodeling phase (3-18 months): This occurs over a longer period of time and involves ECM protein degradation and re-synthesis. There is a shift to fewer cells and an increase in the amount of ECM deposited between 9 and 18months (Fig 2F). After this time, only ~15% of the neointimal volume is comprised of SMCs compared with ~60% between 3 and 9 months [68] . During this period hydrated proteoglycans (hyaluronan and versican) and Type III collagen are replaced by decorin and Type I collagen, which can lead to shrinkage of the entire artery, a phenomenon known as negative remodeling [68, 69].

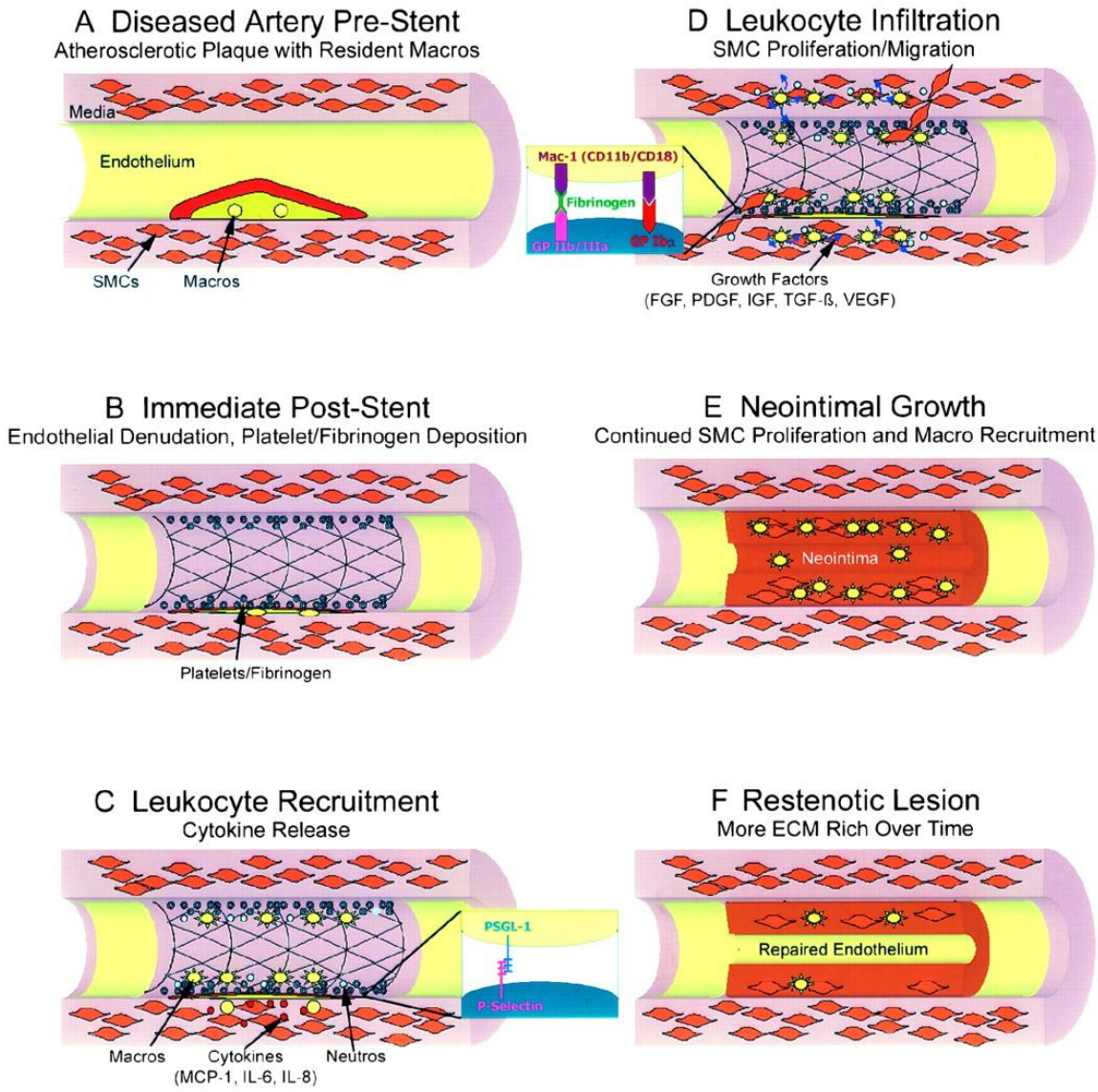


Fig 2. The cascade leading to restenosis [49]. A, Mature atherosclerotic plaque before intervention. B, Immediate result of stent placement with endothelial denudation and platelet/fibrinogen deposition. C and D, leukocyte (neutrophils [neutros] and macrophages [macros]) recruitment, infiltration, and SMC proliferation and migration in the days after injury. E, Neointimal thickening in the weeks after injury, with continued SMC proliferation and monocyte recruitment. F, Long-term (weeks to months) change from a predominantly cellular to a less cellular and more ECM-rich plaque.

1.4 Role of the endothelium in intimal hyperplasia

In addition to regulating vascular tone and permeability, the endothelium is emerging as a key modulator of the cellular response to vessel wall injury [70, 71]. A normal functional endothelium suppresses intimal hyperplasia by inhibiting thrombus formation and inflammation, that trigger intimal hyperplasia, and by reducing SMC proliferation and migration (Fig 3).

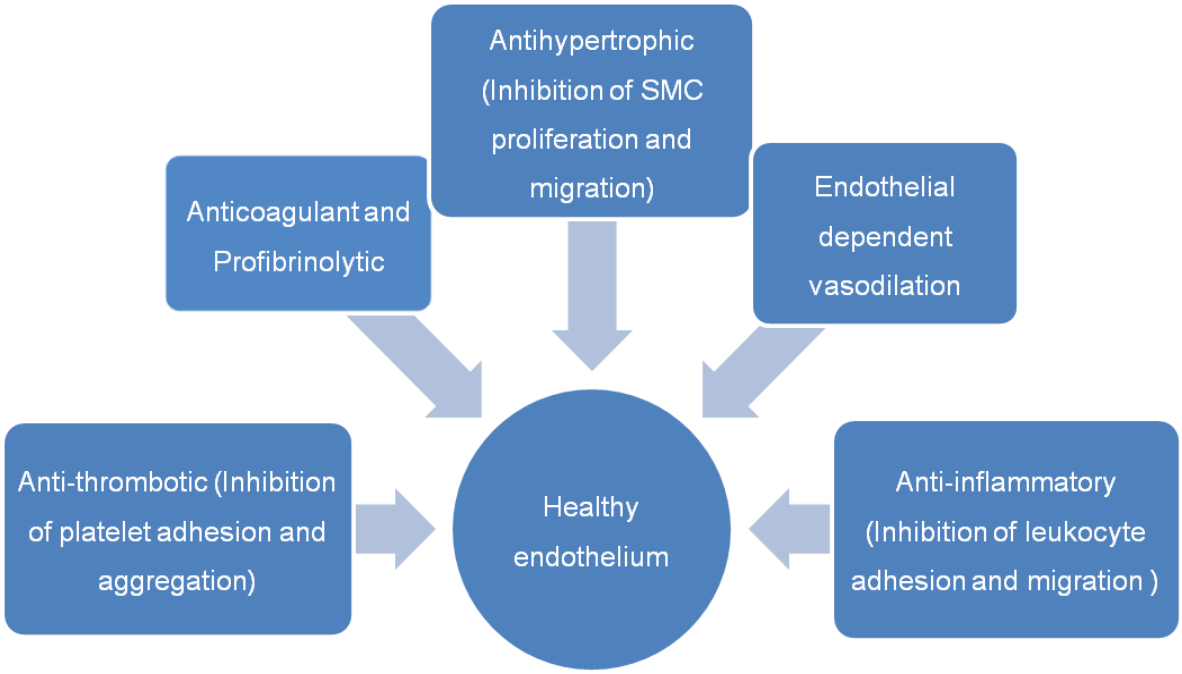


Fig 3. The properties of a healthy endothelium that attenuate intimal hyperplasia

1.4.1 Inhibition of thrombosis and inflammation

The endothelium maintains an antithrombotic environment on its luminal surface by secreting factors that inhibit platelet activation, adhesion and aggregation, such as nitric oxide (NO) and prostaglandin I_2 (PgI_2), and by expressing anticoagulant and fibrinolytic activity such as heparin, protein C and S, tissue factor pathway inhibitor (TFPI) and tissue plasminogen activator (tPA) [72]. Nitric oxide also has anti-inflammatory actions through the inhibition of cytokine secretion and adhesion molecules expression[73]. Mice deficient in endothelial nitric oxide synthase (eNOS) have markedly increased endothelial-leukocyte adhesion and accelerated atherosclerotic lesion formation [74].

1.4.2 Inhibition of SMC proliferation and migration

The endothelium inhibits SMC growth by acting as a selectively permeable barrier which protects against circulating growth factors [42]. Endothelial cells also secrete NO, transforming growth factor-β (TGF-β), heparin and heparin-like molecules, which maintain SMCs in a quiescent state [75, 76]. The secreted heparin also binds and neutralises the mitogenic action of FGF [77] . The importance of NO in the control of vascular healing by attenuating vascular inflammation and inhibiting SMC proliferation and migration has been demonstrated in a rat carotid artery injury model [78]. Endothelial nitric oxide synthase (eNOS) gene transfection not only restores NO production in rat carotid arteries after endothelium denudation, but also increases the vascular reactivity of the injured vessels. This treatment leads to a 70% reduction in neointima formation after balloon injury.

1.4.3 Characteristics of a dysfunctional endothelium

Endothelial dysfunction is characterised by a reduction in antithrombotic activity of the endothelium (the production of antiplatelet agents such as NO and prostacyclin, anticoagulants such as TFPI and pro-fibrinolytic agents such as tPA)[72], and an increase in prothrombotic activity of the endothelium through increased expression of procoagulatory mediators such as plasminogen activator inhibitor 1 (PAI-1)[72]. This results in a vascular environment, which together with exposure of highly thrombogenic substances from ruptured or erosive plaques, incites thrombus formation.

Dysfunction of the endothelium also results in an imbalance between vasodilator substances with antiproliferative properties (e.g NO) and vasoconstrictors with mitogenic properties (such as endothelin, thromboxane A_2, prostaglandin H_2 and reactive oxygen species) that are detrimental to the arterial wall[72,79]. For example, arterial injury in a porcine coronary stent model leads to the aberrant release of endothelin-1, and treatment with an endothelin receptor antagonist, reduces the intimal hyperplasia that normally ensues [80]. The systemic and local milieu associated with endothelial dysfunction therefore favours cell proliferation, intimal hyperplasia, and vasoconstriction, which may contribute to the restenosis process.

1.4.4 Clinical implications of impaired endothelial function

Endothelial dysfunction is considered to be a systemic process [81, 82], with endothelial activity in a peripheral artery reflecting systemic function. Endothelial function is assessed in vivo by measuring flow mediated dilatation (FMD), most commonly in the brachial artery [83]. The change in the diameter of the artery is assessed by inflating a cuff around the arm to produce shear-induced endothelial nitric oxide.

Direct measurement of coronary endothelial function involves local delivery of acetylcholine via coronary catheter followed by video assisted measurements in coronary calibre [84]. Acetylcholine causes a dose-dependent dilation of coronary arteries in subjects without coronary disease, whereas in patients with coronary disease, a "paradoxical" vasoconstriction is observed, indicating an impaired endothelium-dependent vasomotion [85]. This vasoconstriction also occurs in a progressive manner depending on the degree of endothelial dysfunction [70]. The invasive nature of this technique has resulted in its restricted use.

Endothelial dysfunction is observed in the presence of major cardiovascular risk factors, including aging, smoking, diabetes mellitus, hypercholesterolemia and hypertension[86]. The presence of multiple risk factors is associated with a progressive worsening of endothelial function in a step wise manner [87]. Cardiac events in patients with mildly diseased coronary arteries only occurred in those patients showing the lowest tertile of coronary responses to acetylcholine [88].

Patients with impaired endothelial and endothelium-independent coronary vaso-reactivity as shown by increased vasoconstrictor responses to acetylcholine infusion and cold pressor testing, have a significantly higher incidence of cardiovascular events[84]. They also have significantly blunted vasodilator responses to increased blood flow and the intracoronary injection of nitroglycerin[84].

Given the systemic nature of endothelial dysfunction, the question arises as to whether peripheral vascular function may also serve as a prognostic marker. Clinical studies have now addressed this question and have demonstrated that peripheral endothelium-dependent vasodilation, measured in response to acetylcholine or as flow-dependent vasodilation, has profound and independent prognostic implications[83, 89]. Prospective analysis of brachial artery vasodilation in patients undergoing vascular surgery[83], shows that preoperative, flow-dependent, endothelium-mediated dilation is significantly lower in patients with a postoperative event, compared with those without an event, and this is an independent predictor of outcome. Analysis of forearm blood flow in response to acetylcholine in patients with coronary disease [89] showed that the increase in forearm blood flow in response to acetylcholine was an excellent prognostic indicator i.e. the subsequent event rate was high in those with blunted responses to acetylcholine.

The relationship between endothelial function and acute vascular injury has been investigated in patients with single-vessel CAD undergoing percutaneous coronary intervention (PCI) with bare metal stenting[71]. Flow mediated dilation (FMD), assessed 30 days after PCI was significantly impaired in patients with restenosis

versus those without restenosis with 4% of patients with FMD ≥ 7% developing in-stent restenosis versus 28% of those with FMD <7%. Multivariate analysis revealed that FMD was the strongest independent predictor of restenosis.

Measurement of FMD in the brachial artery before and six months after PCI in patients who had elective PCI [90] revealed that an impaired FMD at follow-up was strongly associated with restenosis, independent of other clinical and angiographic variables known to be associated with restenosis. Initial FMD was not associated with subsequent restenosis.

These studies demonstrate that the endothelium is important in regulating the local response to acute and chronic vascular injury, and that endothelial dysfunction may exacerbate neointima formation. Enhancing re-endothelialisation and endothelial function after injury may therefore have the potential to reduce the formation of neointima.

1.5 The bone marrow as a source of neointimal cells

Until a decade ago, it was thought that the formation of postnatal blood vessels as well as the neointima was exclusively dependent on the proliferation and migration of local endothelial and vascular smooth muscle cells[91]. It has since been hypothesized that, as haematopoietic stem cells (HSCs), derived from the bone

marrow, are responsible for the replenishment of hematopoietic blood cells, there is a similar stem or progenitor cell, termed an Endothelial Progenitor Cell (EPC), that regenerates the endothelium and participates in vessel formation[92]. There have been a number of studies in animal models and man to support this concept.

1.5.1 Bone marrow transplant studies in animals

The contribution of bone marrow derived cells to the neointimal lesion has been demonstrated using bone marrow transplant in conjunction with mouse models of vascular injury [93-96]. One of the earliest studies of this phenomenon used bone marrow cells expressing β-galactosidase (the product of the LacZ gene, easily identified as blue following addition of the substrate, X-gal to the tissue section), from ROSA26 mice transplanted into wild-type mice in which arterial injury was subsequently induced[94]. Histological analysis of the neointimal lesion at specified time points revealed the presence of LacZ positive, or transplanted bone marrow derived cells. Some LacZ positive cells expressed antigens that identified them as endothelial cells (CD31) while others expressed SMC markers (α-smooth muscle actin). A number of subsequent studies have confirmed the existence of bone marrow derived cells in the neointima, with 20% to 66% of the total neointimal cell population suggested to be of bone marrow origin [93-96]. This wide range could be the result of the different methods of labelling bone marrow cells (BMCs) as well as different types of injury induced. Similar results have been found in animal vein graft models [94, 97] and transplant associated intimal hyperplasia [94, 98].

1.5.2 Human studies

The evidence regarding the origin of neointimal cells in humans is limited. Data obtained from human coronary atherectomy tissue from in-stent and post angioplasty restenosis, and primary atherosclerotic lesions, as well as postmortem coronary artery cross sections from young individuals without atherosclerosis appear to support the concept that these cells contribute to the neointima as suggested by the data from animal models. Between 2% and 30% of the neointimal SMC cells from all restenotic tissue specimens contained cells that expressed the progenitor cell marker c-kit[99] and smooth muscle α-actin, whereas the intima and media of primary athersclerotic lesions and normal arteries were devoid of c-kit-expressing cells [100]. This supports the animal studies in showing that bone marrow derived cells contribute significantly to neointima formation.

1.5.3 Endothelial progenitor cells

Asahara et al. [101] hypothesised that an endothelial progenitor cell (EPC) may express cell surface markers shared by HSCs, since endothelial and haematopoietic cells share a similar mesodermal origin during embryonic development. Placing CD34+ cells (15.7% enriched) on fibronectin coated plates results in the formation of spindle shaped cells that express a variety of proteins such as CD31, vascular endothelial growth factor receptor (VEGFR-2 or Flk-1), Tie-2 and E-selectin that are also expressed by endothelial cells[101]. Injection of

$CD34^+/VEGFR2^+$ cells into a mouse model of hind-limb ischaemia results in the incorporatation of these cells into sites of neovascularisation in the affected limb. $CD34^+$ cells, isolated from peripheral blood, umbilical cord blood and bone marrow[92, 101] and injected into a variety of pathological conditions, are recruited to foci of neovascularisation, such as ischaemic skeletal muscle[101-104] and myocardium [101, 102, 105], tumours [102, 106], as well as areas of endothelial denudation[107, 108]. These cells contribute to neovascularisation and neoendothelialisation through their ability to differentiate into mature endothelial cells, incorporate into blood vessels; and augment angiogenesis through the secretion of angiogenic growth factors[109, 110]. These studies provided the first direct evidence for the existence of a putative EPC that contributes to vasculogenesis (i.e. de-novo blood vessel formation) post-natally, and challenged the traditional concept that endothelial regeneration occurs exclusively via the proliferation of pre-existing resident vessel wall endothelial cells.

1.5.4 Defining endothelial progenitor cells

Over the past 15 years there have been many studies that have suggested that bone marrow derived EPCs, can differentiate into mature endothelial cells (EC) and maintain endothelial function [111, 112]. However these studies have employed a variety of methods to define EPCs and this has resulted in confusion as to what exactly constitutes an EPC and how to define it. This has stemmed from a lack of a

specific and unique cell surface or molecular marker that would permit prospective isolation of this cell.

1.5.4.1 Phenotypic identification

Many investigators have identified the EPC by fluorescence activated cell sorting analysis (FACS) using monoclonal antibodies directed at a variety of cell surface markers which has resulted in a complicated list of EPC phenotypes (Table 2)[113]. The early phenotypic identification was based on the co-expression of both haematopoietic (CD34) and endothelial (VEGFR2) marker proteins[113]. These two proteins were used in most FACS analysis as earlier studies showed that $CD34^{+ve}$ and $VEGFR2^{+ve}$ cells were able to generate ECs in-vitro[101]. CD34 is expressed on haematopoietic stem cells, but is also expressed on activated endothelium of certain microvessels [114]. VEGFR2 is also expressed on hematopoietic stem cells and mature endothelial cells [115]. In order to separate EPCs from circulating mature endothelial cells, the stem cell marker, CD133 [116], was used to discriminate EPCs from mature endothelial cells that do not express it. Thereafter cells expressing CD34, VEGFR2 and CD133, so called triple positive cells, were widely accepted as defining true EPCs.

Many studies have used CD34, CD133 and VEGFR2 to define EPCs and have found that EPC number correlates inversely with risk of developing cardiovascular disease and prognosis in established disease [117, 118]. Until relatively recently, however, the ability of these triple positive cells to generate ECs in-vitro or in-vivo

had not been proven [116, 119]. A recent study found no evidence that isolated human umbilical cord blood and mobilised peripheral blood $CD34^{+ve}$/$VEGFR2^{+ve}$/$CD133^{+ve}$ cells contribute to the formation of ECs in-vitro[120]. In-vitro haematopoiesis assays have shown that these cells in fact represent an enriched population of CD45+, myeloid hematopoietic precursors[120]. Though it is difficult to know exactly what the significance of this cell type is or to know whether it acts as a true EPC, it clearly has an impact on cardiovascular disease.

Table 2. Surface immunophenotype of human and murine EPCs (reproduced from Timmermans et al.[113]).

EPC immunophenotype in humans	
$CD34^+CD31^+$	$CD34^+$VE-Cadherin$^+CD3^-$
$CD34^+CD62L^+$	$CD34^+CD133^+VEGFR\text{-}2^+CD45^+$
$CD34^+CD133^+$	$CD34^+CD45^+CD146^+$
$CD34^+CD11b^+$	$CD34^+CD45^-$
$CD34^+CD45^+$	$CD133^+$
$CD34^+CD133^+CD45^+$	$CD133^+CD45^-$
$CD34^+$FGFR1$^+$	$CD133^+VEGFR\text{-}2^+$
$CD34^+VEGFR\text{-}2^+$	$CD14^+VEGFR\text{-}2^+$
$CD34^+CD133^+VEGFR\text{-}2^+$	$CD14^+CD34^+$
$CD34^-CD133^+VEGFR\text{-}2^+$	$ALDH^{bright}$
EPC immunophenotypes in mice	
Sca-1^- Lin$^-$ cKit$^-$	cKit$^+CD31^+$
cKit$^+CD34^+$Flk-1	$CD45^-CD34^+$Flk-1^+
Sca-1^+cKit$^+$Lin$^-$	CD31 $^+$Flk-1 $^+CXCR4^+$

1.5.4.2 Colony-forming Unit (CFU) assay

The *in vitro* colony forming unit assays have been used by many to measure EPC number and function[111, 121]. This technique involves the culture of isolated peripheral blood mononuclear cells (PBMC) on fibronectin in endothelial growth medium. This leads to the outgrowth of colonies of endothelial like cells. Two distinct EPC populations have been classified according to the time at which they appear in culture: early EPCs and late outgrowth endothelial cells (OECs) [104] (Table 3). Early EPCs appear in culture after 4-7 days and are similar to those originally described by Asahara et al. [101] while OECs appear much later in culture, after 14–21 days[122]. These two different populations of cells are also phenotypically and morphologically distinct (Fig 4). Early EPCs (CFU) are clearly evident from three days onwards. The emergence of cobblestone shaped cells is seen at around 10-20 days with rapid proliferation thereafter.

Table 3. Characteristics of human EC-like cells and OECs [113] .

EC-like cells	OECs
(EPCs, ECs, CFU-ECs, CACs, early outgrowth CE-EPCs, early EPCs)	(EPCs, ECs, CFU-ECs, OECs, ECFCs, EC-like, late EPCs, late endothelial outgrowth)
1. Generated after 4–21 days in culture	1. Appear after > 7 days in culture
2. Round (pancake) to spindle shaped appearance; no typical confluent monolayer	2. Typical polygonal cells in a confluent cobblestone monolayer
3. Express endothelial and haematopoietic markers (e.g. CD45, CD14)	3. Express CD31, CD34, CD105, CD146, VE-Cadherin, VEGFR-2, but not the haematopoietic surface markers CD133, CD14 or CD45
4. Bind UEA-1 lectin and take up LDL	4. Bind UEA-1 lectin and take up LDL
5. Maintain haematopoietic potential and/or functions	5. Have no apparent haematopoietic potential
6. Have low proliferative potential	6. Bear high proliferative potential
7. Do not generate vascular tubes *in vitro* in matrigel	7. Generate vascular tubes *in vitro/in vivo* in matrigel
8. Improve neovascularization *in vivo*	8. Improve neovascularization *in vivo*
9. Originate from CD45+ haematopoietic lineage cells ($CD34^+CD45^+$, $CD133^+CD45^+$, $CD34^-CD45^+$, $CD14^+CD45^+$)	9. Originate from $CD45^-CD133^-CD34^+$ cells, bone marrow and the vascular wall

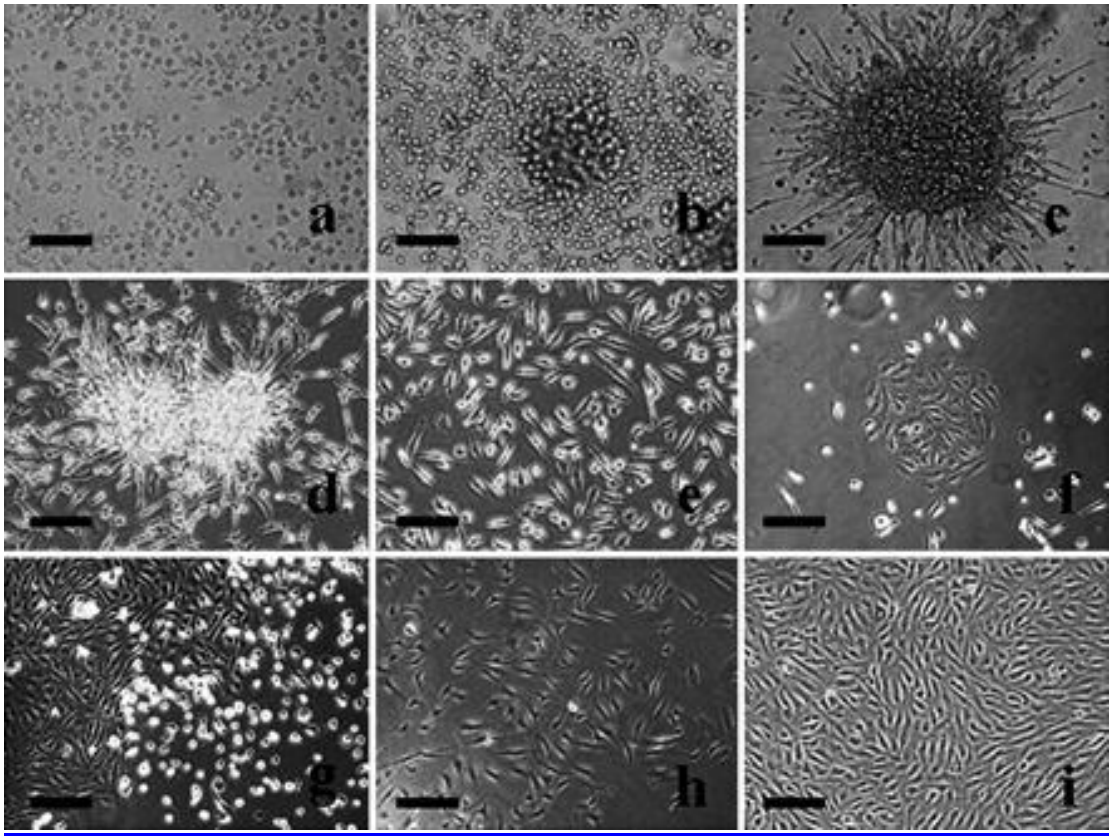


Fig 4. Sequential changes of cultured EPCs after seeding PBMCs.

a) PBMCs immediately after plating; (b) 1 day; (c) 3 days showing a typical early CFU; (d and e), 10 days; (f) 21days; (g-i) OECs grow exponentially to confluence showing a cobblestone-like monolayer. (From Hur et al 2004[104]).

Early EPCs express hematopoietic-specific cell surface antigens CD14 and CD45, have lower capacity for EC marker expression, and appear as elongated cells in isolation or in colonies. Conversely, OECs can be distinguished from early EPCs by their lack of expression of CD14 and CD45 and their endothelial-like cobblestone morphology. Another important difference between these putative progenitors is their growth rate in culture. Early EPCs display little if any

proliferation, while OECs have high proliferative potential, being capable of over 100-fold expansion *in vitro*. Because of this proliferative ability, OECs are now considered by some to represent true endothelial progenitors. A hierarchy of late OECs has been identified and characterized according to their proliferative potential[121].

Early EPC and OECs also show different angiogenic properties[123]. Early EPCs are known to secrete a variety of angiogenic growth factors such as VEGF, G-CSF, GM-CSF and hepatocyte growth factor[109, 110], but fail to independently form tubules or incorporate into differentiated EC tubules. They do however, augment tubulogenesis by differentiated ECs in a paracrine manner, even when physically separated by transwells[123]. By contrast, OECs independently form tubules and incorporate into differentiated EC tubules, but do not exert a significant paracrine angiogenic effect[123]. A recent study of various bone marrow-derived cells demonstrated specific expression of eNOS by putative EPCs, suggesting this may be a means by which to identify endothelial progenitors[124]. However, eNOS expression has also been shown in a variety of cells including CD14 and CD45 positive cells as well as embryonic stem cells [125].

Despite the obvious in vitro distinction between early and late outgrowth cells, their in vivo or clinical significance remains unclear. Most studies have measured early EPC colonies and it was using this method that an inverse relationship between EPC number and senescence and cardiovascular risk was first established[111].

When transplanted these cells were thought to replace damaged endothelium and/or contribute to neovascularisation[106, 126, 127]. However, recent in-vivo studies have reported that early EPCs enhance neovascularisation and endothelialisation in an indirect paracrine manner[109, 128]. This would reconcile data from earlier studies that show limited incorporation of early EPCs into newly formed vessels despite enhanced neovascularisation[129]. In contrast, late outgrowth cells have not been well studied in vivo, in part due to difficulties in developing a reproducible assay[104]. In animal models OECs have been shown to augment early re-endothelialisation and thus attenuate intimal hyperplasia following wire injury[130, 131]. It has been suggested that early EPC and OECs may operate in a synergistic fashion to enhance neovascularisation and re-endothelialisation[110]. The different angiogenic properties of early EPCs and OECs suggests that different strategies may be required to optimally enhance their respective therapeutic potential.

1.5.5 Identification of endothelial cells

Identifying a cell as a true EC is crucial before its precursor cell can be classified as a true EPC. To identify ECs generated from EPCs in vivo, most reports have used a single marker or combination of cell surface markers that are typically expressed on normal vascular ECs. These include CD31, CD34, VEGFR2 and Tie-2 and the uptake of acetylated low density lipoprotein (acLDL) and binding of a plant lectin - Ulex Europaeus Agglutinin-1 (UEA-1, Fig 5) [101, 111, 132]. There is however significant overlap in the antigens expressed by various cell types. Hematopoietic stem cell populations express both CD34 and CD31 as well as a

number of other traditional endothelial markers including VEGFR2 and Tie-2 [113]. A number of studies have also shown co-expression of a raft of other endothelial antigens, as well as the uptake of acLDL and the binding of UEA, by circulating cells that express myeloid markers such as CD14 and CD45[125, 133].

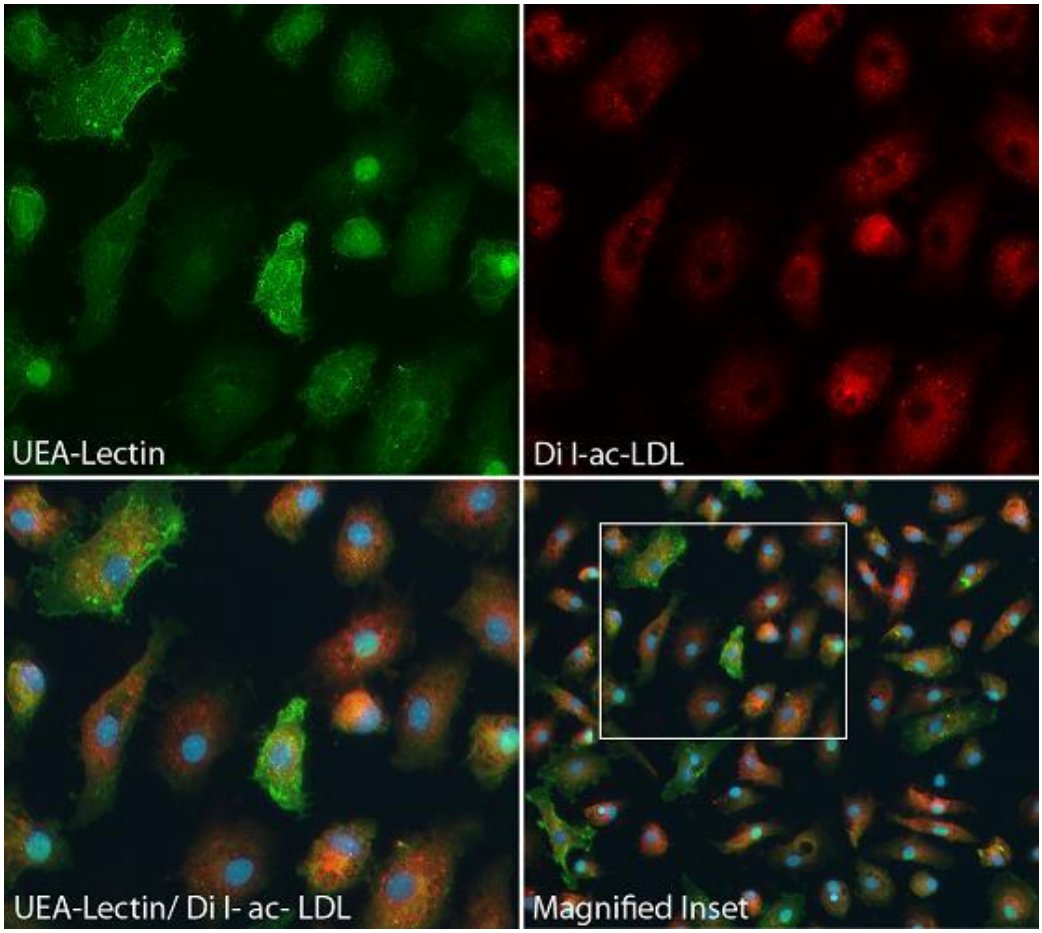


Fig 5. Characterization of EPCs : lectin-binding, LDL-uptake. EPCs exhibit *UEA*-1 lectin-binding (green) and acLDL-uptake (red). Merged image of both stains with blue nuclei in the bottom panels.

1.5.6 Comparison between EPC measuring techniques

The differing ways of measuring EPCs have also been found not to correlate with each other [134]. CFU assay numbers do not correlate with flow cytometric detection using antibody combinations $CD34^{+ve}/VEGFR2^{+ve}$ or $CD34^{+ve}/CD133^{+ve}/VEGFR2^{+ve}$, but correlate negatively with $CD34^{+ve}/CD133^{+ve}$ numbers. $CD34^{+ve}/VEGFR2^{+ve}$ numbers correlate with $CD34^{+ve}/CD133^{+ve}/VEGFR2^{+ve}$, but not with $CD34^{+ve}/CD133^{+ve}$. It is therefore likely that the methods used to distinguish an EPC measure a heterogeneous group of cells/progenitors that consist of a number of phenotypes. Interpretation of data from flow cytometry or in vitro CFU assay therefore makes comparison of data between laboratories difficult, and may account for the discrepancies in the results from the different studies. It is evident that the field has suffered from the use of a single term, EPC, to refer to BM-derived or circulating cells of diverse lineages. In light of this we have adopted the term haematopoietic progenitor cell (HPC) when describing cells that have been historically termed EPC except when studies have specifically used the OEC assay. The variety of cells that have been shown to share similar properties to HPCs, as well as the difficulties in defining them has led some to suggest a functional classification [135, 136]. The cells must:

- have the ability to differentiate into and function as endothelial cells.
- contribute to post - natal vasculogenesis and/or vascular homeostasis.
- demonstrate characteristics of stem cells i.e proliferative capacity.

1.5.7 HPCs, vascular repair and endothelial function

1.5.7.1 Evidence from animal studies

HPCs form part of an endogenous endothelial repair mechanism that attenuates vascular damage by maintaining endothelial function [111, 112]. Direct incorporation of circulating HPCs into injured vessels has been observed in animal models of arterial injury[137, 138] and following arterial and vein graft transplantation[97, 139] . In a model of transplant atherosclerosis, regenerated endothelial cells from arterial grafts are found to originate from recipient circulating blood and not the remaining endothelial cells of the donor vessels [139]. Similarly, the endothelial monolayer in a vein graft model which is completely lost 3 days post surgery, is subsequently replaced by circulating endothelial progenitors[97].

HPCs are also thought to mediate vascular repair and attenuate atherosclerosis progression even in the continued presence of vascular injury. Chronic treatment with bone marrow-derived progenitor cells from young non-atherosclerotic ApoE knockout (ApoE KO) mice prevents the development of the disease in aged ApoE KO recipients despite persistent hypercholesterolemia[140]. Although the mechanisms involved are still not clear, HPCs appear to contribute to the restoration of the endothelial monolayer[140].

In addition to bone marrow, spleen-derived HPCs also have the capacity to repair damaged endothelium. Intravenous infusion of spleen-derived HPCs improves

endothelium-dependent vasodilatation in atherosclerotic mice, indicating that progenitor cells play an important role in repairing the vascular injury [141].

1.5.7.2 Evidence in man

Reduced endothelial function occurs in a progressive fashion as the number of atherosclerotic risk factors increases[87] and each of these risk factors is associated with reduced HPC number and function[111, 117, 119, 142-144]. For example, HPCs from type-II diabetic patients exhibit impaired proliferation, adhesion and reduced angiogenic potential in vivo[144]. Similarly there is reduced survival, migration and proliferation of HPCs in aged individuals compared with younger subjects[145].

HPC numbers can also be used as a predictive biomarker for cardiovascular risk and vascular function. Patients at highest risk of developing cardiovascular disease (Framingham score) have fewer HPCs compared with their low-risk counterparts[111]. HPCs isolated from patients with existing coronary artery disease also have reduced levels and migratory activity compared with healthy individuals, and this is inversely correlated with the number of risk factors[117].

Circulating HPCs are negatively associated with the degree of carotid athersclerosis[146] and in patients who develop coronary artery disease, there is a significant association between increasing numbers of HPCs and decreased risk of a major cardiovascular events and hospitalisation[118].

HPC numbers also inversely correlate with endothelial function in patients with coronary heart disease[147] and healthy individuals[111], although it has been suggested that the functional capacity of HPCs (e.g. ability to migrate towards a chemotactic stimulus) may be more important than their number in maintaining endothelial function[145].

HPCs and endothelial dysfunction are, therefore, thought to link cardiovascular risk factors and clinical events. HPC numbers rise acutely immediately after coronary artery bypass grafting [148, 149] and angioplasty [150] as well as after acute myocardial infarction [151], further supporting the concept that they form an important part of the acute response to endothelial damage and ischaemia. Low HPC numbers are associated with the most severe neurological impairment in patients who have had a stroke, and are also an independent predictor of poor prognosis[152]. These data suggest that HPCs are not only involved in cardiovascular disease development, but also with disease progression and subsequent prognosis.

1.5.8 HPCs and the inhibition of neointima formation

Patients who developed restenosis (defined as >40% stenosis) have a decreased number and increased senescence of HPCs compared with patients without restenosis[153]. Both of these groups also have fewer circulating HPC numbers than in patients with no coronary artery disease[100], but increased number of senescent HPCs appears to be the only independent factor associated with in-stent restenosis[153]. Patients with angiographically-demonstrable diffuse in-stent

restenosis have reduced numbers of circulating HPC when compared with those developing focal restenosis[154]. There is also impaired adhesion of HPCs in patients with restenosis compared with patients who have a patent stent[154].

Increasing the number and function of HPCs might have therapeutic potential as a means of augmenting the restoration of endothelial integrity and function. Physical exercise increases circulating HPC numbers and reduces neointima formation after carotid injury in mice [155]. Exercise is also associated with a rise in HPC numbers in humans [156].

Following transplantation of bone marrow transfected by a retrovirus with enhanced green fluorescent protein in a mouse model of neointima formation, up to 10% of neointimal endothelial cells (vWF^+ cells) after carotid wire injury were also GFP^+. This supports the notion that bone marrow-derived progenitor cells may contribute to re-endothelialisation following vascular injury[108]. Enhancing the circulating pool of endothelial progenitor cells by administering a statin, increases the appearance of bone marrow-derived cells (BMCs) in the injured vessel wall, accelerates re-endothelialisation and significantly decreases neointimal formation[108]. Mouse spleen-derived mononuclear cells also differentiate into cells with characteristics of HPCs. Intravenous injection of these spleen-derived HPCs after wire carotid injury accelerates re-endothelialisation and decreases neointimal hyperplasia, suggesting that HPCs have an important role in regulating neointimal formation[138]. Infusing culture-expanded autologous HPCs[138,157], or enhancing their

mobilisation following injury with statins[137], granulocyte colony stimulating factor (G-CSF)[158,159] or granulocyte macrophage-CSF[107] also enhances re-endothelialisation and decreases neointima formation in animal models of arterial injury.

Enhancing the numbers of circulating progenitor cells has not, however, always proved beneficial. Injecting BMCs into ApoE$^{-/-}$ mice, following induced hindlimb ischaemia, not only increases neovascularisation to these oxygen deficient regions, but also accelerates plaque formation and lesion size when compared with control groups[160]. Treatment with BMC-derived HPCs results in increased lesion size compared with controls, while plaques have larger lipid cores and thinner fibrous caps. Reduced levels of the anti-inflammatory cytokine, IL10 are also seen in the atherosclerotic aortas[161]. This may be the result of the proinflammatory properties of these cells[161]. Similarly, even though implantation of an arteriovenous anti-CD34-ePTFE graft in pigs resulted in rapid endothelialisation within 72 hours and persistent endothelial graft coverage, intimal hyperplasia at the outflow tract was profoundly increased at 4-weeks after implantation when compared with bare stents[162]. Besides the differences between various experimental models, it is difficult to reconcile these findings. Overall, it seems that excessive mobilisation of HPCs may lead to restenosis, whereas an absence of these cells may impair re-endothelialisation (Figure 6).

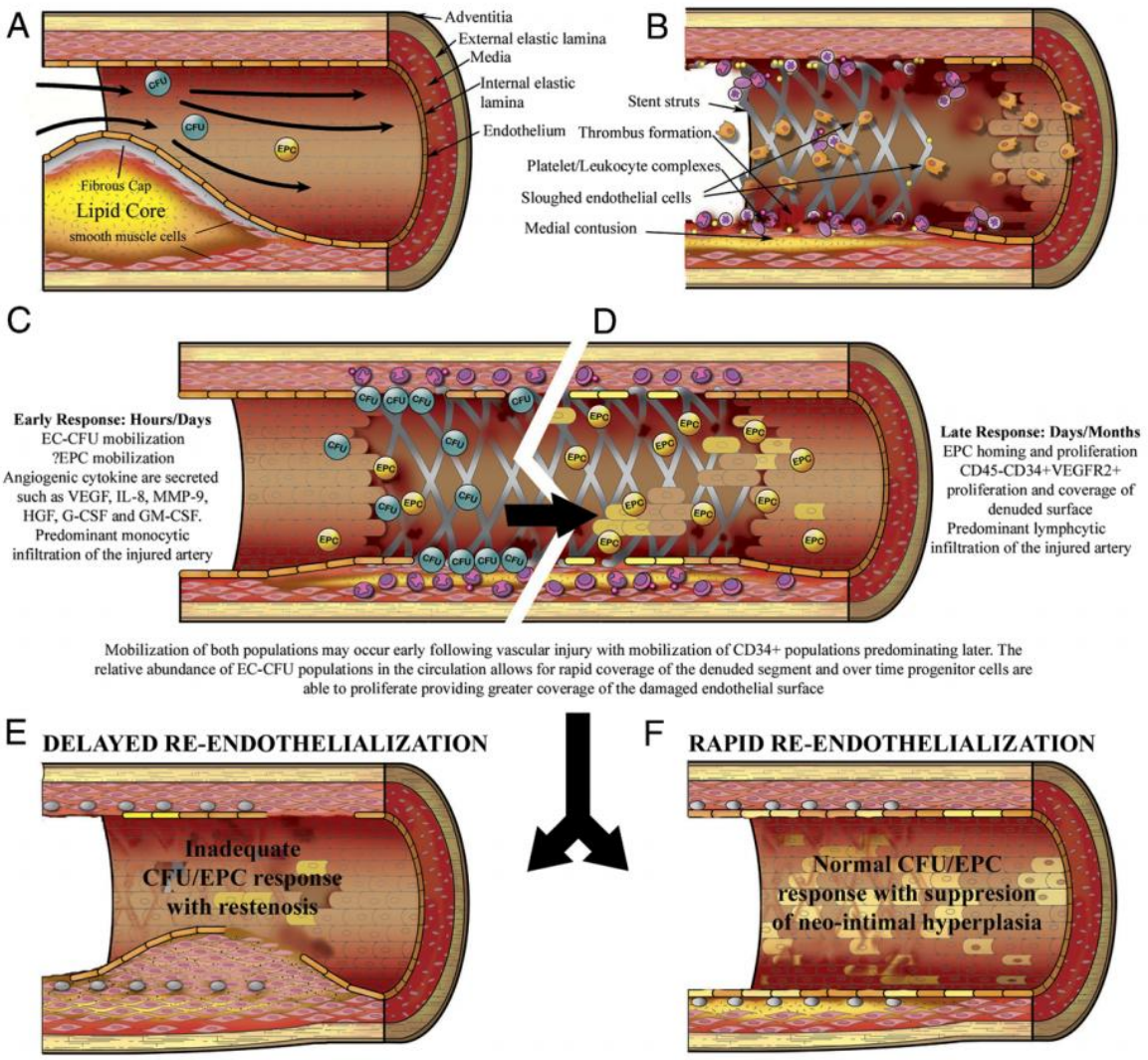


Fig 6. Putative role for circulating progenitor cells after arterial injury[163].

(A) Under resting conditions, circulating concentrations of the precursors of endothelial cell colony-forming units (EC-CFUs) and circulating endothelial/haematopoietic progenitor cells (EPCs) are low, particularly in patients with atherosclerotic disease. **(B)** Intra-arterial injury/stent placement initiates the inflammatory cascade. **(C)** The early response consists of mobilization of the precursors of EC-CFUs involving angiogenic monocytes and lymphocytes. EC-CFU precursors home to the site of injury and avidly secrete angiogenic factors,

encouraging resident endothelial cell proliferation and migration and the mobilization and homing of bone marrow-derived and local endothelial progenitors to the site of vascular injury. **(D)** Over a period of weeks and months, progenitor cells proliferate, contributing to effective re-endothelialization and the restoration of vascular homeostasis. **(E)** An inadequate EC-CFU/EPC response may lead to delayed re-endothelialization and persistent inflammation, thus potentiating smooth muscle hypertrophy and extracellular matrix deposition, leading to restenosis. **(F)** A robust response by the bone marrow leads to rapid homing of EC-CFU precursors to sites of vascular injury with recruitment and integration of HPCs to the endothelial monolayer, facilitating rapid re-endothelialization and recovery of normal endothelial function and vascular homeostasis. CFU = colony-forming unit; G-CSF = granulocyte-colony stimulating factor; GM-CSF = granulocyte-macrophage- colony stimulating factor; HGF = hepatocyte-like growth factor; IL = interleukin; MMP = matrix metalloproteinase; VEGF = vascular endothelial growth factor; VEGFR2 = vascular endothelial growth factor receptor-2.

1.5.9 HPCs and restenosis : clinical trials

Stem cells and in particular HPCs have been the subject of a number of clinical trials attempting to reduce the risk of restenosis. These trials were motivated by the early animal studies which showed only the beneficial effects of enhanced HPC numbers on restenosis. The HEALING-FIM and HEALING II studies (Healthy Endothelial Accelerated Lining Inhibits Neointimal Growth – First In Man) [25, 164, 165] used a stent coated with the monoclonal antibody to CD34, a progenitor cell marker, to capture circulating HPCs. These studies were the first to clinically evaluate the results of an HPC capture stent but did so in de novo single coronary lesions in a non randomised setting. They showed that the use of such a stent is safe and feasible. This was also subsequently demonstrated in patients undergoing angioplasty with high risk angiographic or clinical features [166] and in patients undergoing primary angioplasty for myocardial infarction [167]. No patients in these studies showed evidence of late stent thrombosis, although a subsequent study reported a patient receiving an HPC capture stent developed stent thrombosis 5 months after implantation [168]. This suggested that until further data is available as to the incidence of late stent thombosis, there is a need for prolonged double anti-platelet therapy post-angioplasty in patients receiving the HPC capture stents.

Randomised trials are lacking in this area. The TRIAS study (TRI-stent Adjudication Study), which is a multicentre, randomised controlled trial comparing

the HPC capture stents with bare metal stents in lesions at low and high risk of restenosis is currently underway to redress this problem [169].

The safety of two methods of enhancing the progenitor cell response to vascular injury has been assessed in the MAGIC cell trial[170]. Patients with acute myocardial infarction were randomised in to receiving granulocyte colony stimulating factor (G-CSF) injections for 4 days, with or without intracoronary re-infusion of peripheral blood stem cells harvested before angioplasty and stenting. Enrolment was stopped after they found an unexpectedly high rate of restenosis in patients who received G-CSF compared with the control group. The investigators recognised the possibility that progenitors may have differentiated along a smooth muscle phenotype. This finding prompted follow-up studies of how G-CSF might affect neointimal hyperplasia in animal models. In a model of rabbit iliac artery stenting, 60 days after stenting rabbits treated with G-CSF developed significantly more intimal hyperplasia when compared with rabbits treated with placebo [171]. At early time points after injury, treatment with G-CSF not only increased total peripheral white blood cell count, but specifically increased the number of putative HPCs ($CD31^{+ve}$, VE-$cadherin^{+ve}$, $CD34^{+ve}$, KDR^{+ve} cells) and smooth muscle progenitor cells (VE-$cadherin^{+ve}$/a-SMA^{+ve} or $CD31^{+ve}$/α-SMA^{+ve} cells). Culture of these cells over 3 weeks with VEGF resulted in these cells exhibiting an endothelial cell phenotype (cobblestone shape and CD31 positivity); while treatment with PDGF resulted in a smooth muscle like cell phenotype (hill and valley morphology and α-SMA positivity). Increasing the number of progenitor cells may therefore be

beneficial to some types of injury (myocardial infarction), but may exacerbate others (arterial injury). These studies also highlight the risk of using a relatively non-specific factor to stimulate progenitor cell mobilisation.

1.6 Other sources of neo-endothelium

In addition to HPCs, there are a growing number of cell types with the potential to enhance endothelialisation, but with differing properties and origins.

1.6.1 Mesenchymal stem cells (MSCs)

MSCs are bone marrow and tissue-derived multipotent cells capable of differentiating into a variety of tissues including bone, cartilage, muscle and connective tissue [172-176]. These progenitor cells also have the capacity to differentiate into cardiomyocytes[177], endothelial cells (ECs)[178, 179] and smooth muscle cells (SMCs)[180] and can be mobilised in response to G-CSF stimulation[132]. Currently, MSCs are one of the cell types being used in clinical trials for post myocardial infarction cardiac regeneration therapy[181, 182]. It has been shown that an intra-myocardial injection of autologous MSCs or intravenous administration of MSCs can increase vasculogenesis and improve cardiac function after myocardial infarction in animal experiments and clinical trials[181, 182].

The role of MSCs in intimal hyperplasia has only been studied in animal models. MSCs exhibit a strong capacity for adhesion to the remodelled vessel wall

undergoing intimal hyperplasia after arterial injury[130], and up to 40% of the neointimal cells may be derived from MSCs [130]. A substantial proportion of MSCs proliferate and differentiate into SMCs and ECs in the neointima[130]. Furthermore, cell therapy with OECs modulates the differentiation of MSCs toward an endothelial-like lineage, leading to early reendothelialisation and attenuation of intimal hyperplasia[130]. Others have reported that MSCs have the potential to differentiate into OECs and these cells subsequently inhibit neointimal hyperplasia by re-establishing endothelial integrity in injured vessels [131]. Following vein grafting in a rat model, engrafted MSCs differentiate into endothelial cells, diminish neointima formation and contribute to the improvement in endothelial function[183]. MSCs, like early HPCs, may mediate cardiovascular repair, via paracrine means. MSCs have been shown to secrete an array of different pro-angiogenic peptides including HGF, insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF), VEGF, and angiopoeitin-1 (Ang-1) [184]. These studies indicate that MSCs play an important role in the repair mechanism following vascular injury and have the potential to attenuate neointima formation.

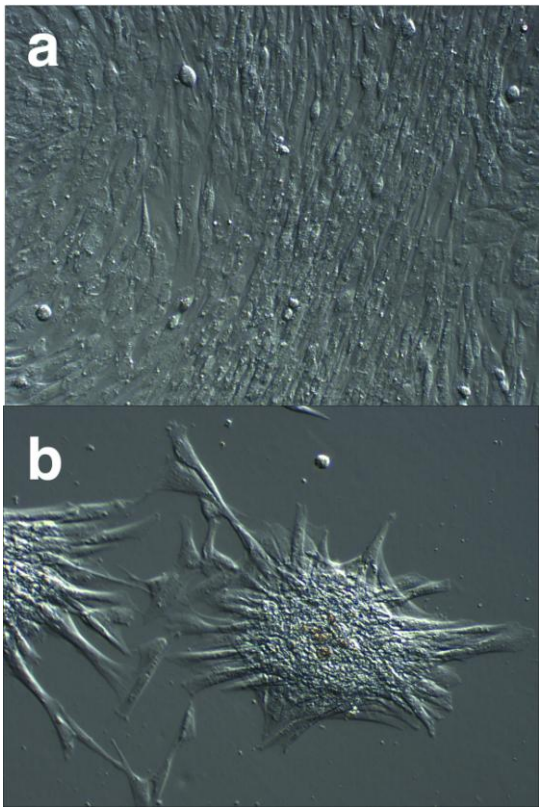


Fig 7. Isolation of mesenchymal stem cells from equine umbilical cord blood[185]. (a) Monolayer of rapidly expanding adherent spindle-shaped fibroblastoid cells compatible with undifferentiated mesenchymal stem cells (× 100). (b) Three-dimensional, relief contrast image of cell cluster of rapidly expanding adherent spindle-shaped fibroblastoid cells compatible with undifferentiated mesenchymal stem cell morphology (× 100).

1.6.1.1 Defining an MSC

MSCs constitute a heterogeneous population of cells, in terms of their morphology, physiology and expression of surface antigens. Up to now, no single specific marker has been identified. MSCs express a large number of adhesion molecules, extracellular matrix proteins, cytokines and growth factor receptors, associated with their function and cell interactions within the bone marrow stroma [186]. The most

commonly detected antigens on MSCs isolated from bone marrow are:- CD105 (SH2; endoglin), CD106 (vascular cell adhesion molecule; VCAM-1), CD73 (SH3 and SH4), CD90 (Thy-1) and Sca-1 [187-189]. MSCs do not possess markers typical for hematopoietic and endothelial cell lineages such as CD11b, CD14, CD31, CD34, CD133 and CD45 [188]. The absence of CD14, CD34 and CD45 antigens on their surface create the basis to distinguish them from the hematopoietic precursors. Since there are no reliable MSC markers, these cells are often isolated using specific cell culture conditions. Typically, a single-step purification method using adherence to plastic cell culture plates is employed. This results in a population of fibroblast-like cells (Fig 7), which are characterized as MSCs based on their ability to differentiate into multiple mesenchymal lineages (e.g. osteogenic, chondrogenic, myogenic, etc.) [190].

1.6.2 The monocyte/macrophage

There is growing evidence that monocytes play an important role in restenosis. The number of macrophages that are recruited into the injured vessel wall correlates with the volume of neointima in animal models[42]. Blocking or gene knockout of either monocyte chemoattractant protein-1 (MCP-1) or its receptor CCR2, blocks monocyte recruitment and significantly reduces neointimal hyperplasia in animal models[191-193]. Conversely elevated levels of MCP-1 are associated with a greater risk of restenosis in patients undergoing coronary angioplasty[194].

Circulating monocytes are thought to be able to trans-differentiate into endothelial cells at the site of arterial injury[109, 133, 195], augment neovascularisation[196] and inhibit neointimal hyperplasia[195]. This contradicts the reported positive correlation between monocyte recruitment and intimal hyperplasia[191-193]. Methods of culturing early HPCs in vitro may in fact produce cells of the monocyte/ macrophage lineage[109, 197]. Monocytes may therefore have the potential to both enhance neointima formation in their capacity as inflammatory cells and to attenuate neointima formation in their capacity to mimic HPC function.

1.6.3 Tissue resident progenitors

Though the bone marrow seems to be an abundant source of progenitor cells recent evidence suggests that these cells may also reside in a variety of other tissues. Isolated spleen-derived mononuclear cells, pre-selected with an endothelial cell medium, demonstrated endothelial cell characteristics and formed tubular-like structures[138]. These cells could sufficiently enhance re-endothelialisation and diminish neointima formation after carotid artery injury[138]. Bone marrow transplantation in a rat model followed by hind limb ischaemia resulted in non bone marrow-derived, tissue resident progenitors (particularly liver and intestine) contributing to postnatal neovascularization to an extent that was similar to that of bone marrow-derived progenitor cells[198]. Systemic infusion of progenitor cells derived from the perivascular niche in the liver incorporated into

vascular structures and subsequently enhanced neovascularisation and improved blood flow recovery in ischaemic hindlimbs[198].

Stem cells have been isolated from the non-adipocyte fraction of adipose tissue [199, 200]. These cells express both haematopoietic and endothelial markers and enhance neovascularisation in animal models of ischaemia via their capacity to differentiate into endothelial cells and their ability to secrete pro-angiogenic peptides[199-201]. Other cells capable of differentiating into an endothelial cell phenotype include bone marrow derived 'side population' $CD34^{-ve}$ stem cells[126], cardiac tissue resident c-kit positive stem cells[202], skeletal muscle stem cells[203], and aortic adventitial cells[204]. In the latter study chimeric mice that express the LacZ transgene (producing the enzyme β-galactosidase) only in bone marrow cells were used to determine the origin of these cells. No β-gal-positive cells were identified, suggesting that these cells are not derived from the bone marrow. Furthermore aortic adventitial cells can differentiate *in vitro* to endothelial and smooth muscle cells. *In vivo* when applied to the adventitia of irradiated vein grafts they differentiate to smooth muscle cells and migrate to the neointima of the grafts[204]. It has also been demonstrated that the adult vascular wall in humans contains progenitor cells. High numbers of HPCs have been identified in an area between the medial and adventitia layer[205]. A complete hierarchy of HPCs can be derived from human vessel wall and discriminated by their clonogenic and proliferative potential[206]. These studies provide evidence that many organs contain progenitor cells that may serve as a circulating pool of HPCs and that a diversity of HPCs exists in human vessels (Fig 8).

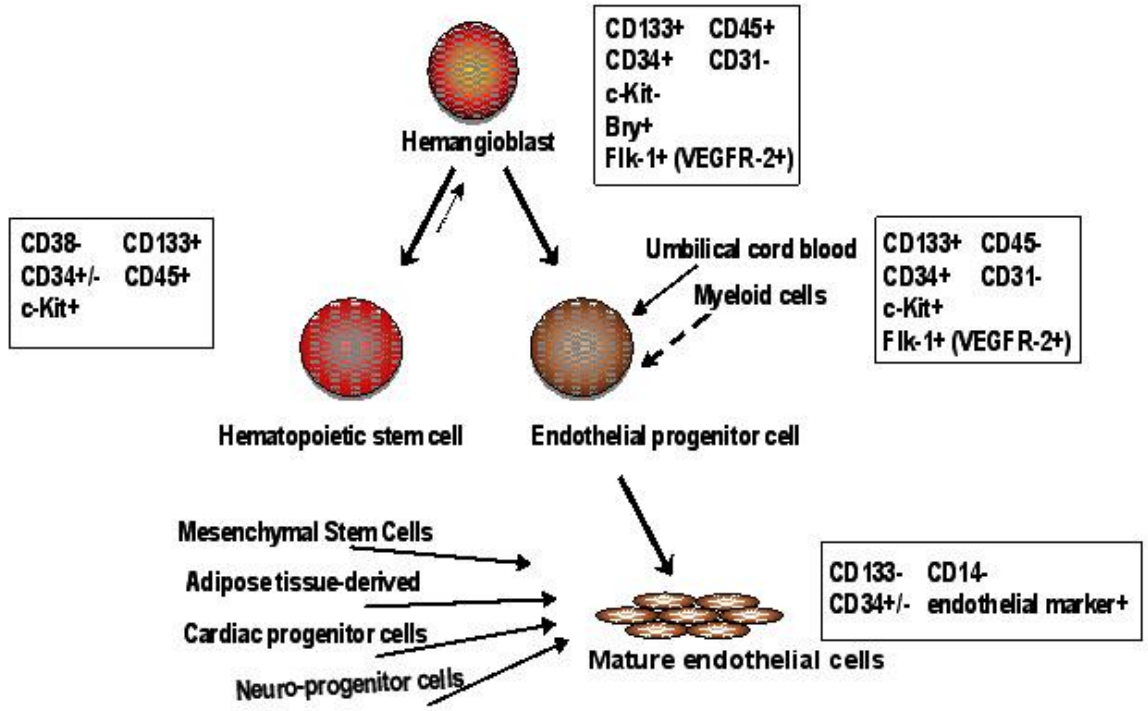


Fig 8. Hypothesised origin, fate and contributors of adult-human endothelial cells with key surface markers[207].

1.7 Smooth muscle progenitors (SMPs)

The proliferation of SMC in response to endothelial denudation is an important component leading to neoimtimal hyperplasia. Neointimal SMCs were thought to originate from the media[91], but, much like the origin for neointimal ECs, this concept has been recently challenged. There is conflicting evidence from animal models regarding the proportion of SMCs that are derived from the bone marrow. Up to 60% of neointimal SMC may be derived from bone marrow progenitors[94, 98],

but when vein segments are grafted, the SMCs that form the neointima are not of bone marrow origin[208, 209]. A possible explanation for this may be that neointimal SMCs are derived from medial SMCs or alternatively from progenitor cells present in tissues other than the bone marrow.

There is now compelling evidence for a bone marrow derived, blood- borne SMP[94, 98, 210, 211], though the exact phenotype of this cell has yet to be defined. Cells present in the bone marrow can express smooth muscle cell antigens after in vitro differentiation[94, 210]. SMCs may also be derived from peripheral blood mononuclear cells when treated with platelet-derived growth factor (PDGF)[211]. These express alpha smooth muscle actin, myosin heavy chain and calponin, as well as CD34, suggesting a bone marrow origin. HPCs are also capable of differentiating into smooth muscle cells[212].

1.8 Progenitor cell recruitment to sites of injury

The recruitment of vascular progenitor cells to sites of neointimal hyperplasia after vascular injury can be conceptualized in three stages: 1) mobilisation of progenitor cells from bone marrow and tissue niches, 2) migration and recruitment of progenitor cells to the site of injury, and 3) differentiation of progenitor cells into mature vascular cells, such as endothelial or SMCs.

1.8.1 Mobilisation of progenitor cells

Stem cell niches are specific sites where stem cells reside in the bone marrow. In these microenvironments the cells can either remain in an undifferentiated and quiescent state or differentiate. In response to vascular injury or physiological stress, stem cells have to be rapidly mobilised and recruited to the damaged area. Progenitor cells are believed to be attached to bone marrow stromal cells or ECM through specific binding interactions, including VLA-4/VCAM-1, SDF-1α/CXCR4, CD44/HA (hyaluronic acid), and interactions between P-, E- and L- selectin[213]. Progenitor cells must migrate through a vascular barrier (bone marrow venous sinuses) that separates the hematopoietic compartment from the circulation in order to exit the bone marrow, and this process is controlled by disruption of these receptor ligand interactions. α_4 and β_2 integrins have a role in the interactions between progenitor cells and the bone marrow microenvironment, and antibodies to these molecules or knock-out models lead to increased progenitor cell mobilization [214, 215]. VCAM-1 is constitutively expressed by bone marrow endothelial and stromal cells, and disruption of the VCAM-1/VLA-4 interaction by antibodies to VCAM-1 or VLA-4 ultimately leads to progenitor cell mobilisation[216, 217].

Vascular endothelial growth factor (VEGF) is thought to be one of the most potent cytokines in stimulating endothelial cell mobilisation, proliferation, migration and survival[103, 218]. Arterial injury models show that augmenting the levels of VEGF

either by local delivery[219] or gene transfer[26, 220] significantly reduces neointima formation and accelerates endothelial regeneration. This effect has not, however, been confirmed in patients undergoing percutaneous coronary intervention[221], where catheter-based intracoronary VEGF gene transfer makes no difference to the restenosis rate. There has been considerable debate over the vasoprotective versus atherogenic effects of VEGF. Extensive vascular networks are well recognised in atherosclerotic plaques[222] and balloon injured arteries[223], raising the possibility that pro-angiogenic agents may exacerbate neointimal growth, in a manner analogous to their role in tumour growth. VEGF also induces migration and activation of monocytes[224], induces adhesion molecules[225] and upregulates monocyte chemoattractant protein -1 (MCP-1)[226], which is also angiogenic.

Granulocyte macrophage colony stimulating factor (GM-CSF) [227], granulocyte colony stimulating factor (G-CSF) [158], stromal derived factor -1(SDF-1) [228], hepatocyte growth factor (HGF) [229] and nitric oxide (NO) [230] all stimulate progenitor cell mobilisation, growth and differentiation. Bone marrow stromal cell expression of eNOS is also essential for the mobilization of stem and progenitor cells [230]. G-CSF can disrupt the CXCR4/SDF-1 complex by signalling the release of proteases from G-CSF receptor bearing neutrophils, most notably neutrophil-elastase, cathepsin-G and matrix-metalloproteinase-9, which then degrades and disrupts CXCR4/SDF-1 interactions [231, 232] and other key adhesion molecules, including VCAM-1.

The SDF-1/CXCR4 interaction is important in regulating progenitor cell survival, cell cycle, and mobilisation[213]. Intravenous administration of exogenous SDF-1α as well as treatment with a specific CXCR4 inhibitor rapidly induces progenitor cell mobilization in both humans and mice [233]. SDF-1, VEGF and G-CSF have all been shown to induce MMP-9 in the bone marrow, leading to cleavage of membrane bound c-kit ligand (mKitL) to soluble KitL. This in turn results in increased progenitor cell cycling and enhanced cell motility, and ultimately leads to progenitor cell mobilization[233, 234]. Cytokine induced progenitor cell mobilisation is dependent on MMP-9 as this process is impaired in MMP-9$^{-/-}$ mice [234]. This study introduced a paradigm in stem and progenitor cell biology whereby activation of a metalloproteinase serves as the decisive checkpoint for the rapid reconstitution of the circulating progenitor cell pool following vascular injury.

1.8.2 Migration to site of injury

Once in the vicinity of an injured vessel, progenitor cells interact with the damaged endothelial monolayer in a similar way as leucocytes interact with activated endothelial cells. Adhesion molecules previously known to be involved in the phase of rolling and firm adhesion of leucocytes are also key regulators of HPC homing. P-selectin and E-selectin seem to mediate the initial steps of this process. Activation of EphB4 in HPCs leads to a higher expression of P selectin glycoprotein ligand-1 (PSGL-1)[235]. Subsequently, increased adhesion to P-selectin and E-selectin is observed. sRNA for P-selectin abrogates this response indicating that PGSL-1 expression facilitates the recruitment of HPCs [235]. Other studies have

shown that E-selectin also mediates HPC-endothelial cell interactions[236]. β2-integrins expressed on the cell surface of HPCs mediate the firm adhesion and transmigration of HPCs to the damaged endothelial monolayer. Activation of the β2 integrins was shown to improve the homing and the neovascularisation capacity of HPCs in a mouse model of hindlimb ischaemia [237].

In addition to playing an essential role in progenitor cell mobilisation, the SDF-1α/CXCR4 axis is also involved in progenitor cell recruitment to sites of injury in models of neointimal hyperplasia [238]. In the mouse wire injury model, blockade of SDF-1α signaling with either a SDF-1α blocking antibody, lentiviral-based local gene transfer of a mutant SDF-1α, or by transplantation of bone marrow cells deficient in CXCR4 results in decreased intimal hyperplasia, which was associated with decreased bone marrow derived neointimal smooth muscle cells [238]. SDF-1α also induces platelet adhesion at the site of injury, and subsequent release of platelet P-selectin led to progenitor cell adhesion and recruitment[238]. These same mechanistic findings suggesting an important role of SDF-1α and platelets in progenitor cell recruitment were demonstrated in vivo using real-time video-fluorescence microscopy[239]. This study also showed that activated platelets secrete high levels of SDF-1[239]. As platelets adhere within minutes to the exposed sub-endothelial surface in injured vessels, this could provide a very effective mechanism of mobilisation and homing of stem cells to the damaged area. In vitro studies have shown that SDF-1α induces HPC and $CD34^+$ cell migration and $CD34^+$ cell adhesion[240, 241]. SDF-1α also has an important role in the recruitment of

HPCs to diabetic wounds in mice [242]. Placental growth factor (PlGF) is known to have potent effects on vasculogenesis and may also be important for HPC function[243, 244]. PlGF stimulates recruitment of HPCs to areas of vasculogenesis[245], though its role in relation to intimal hyperplasia has yet to be studied.

1.8.3 Progenitor cell differentiation

VEGF induces differentiation of both HPCs [103] and MSCs[179] into cells with phenotypic and functional features of endothelial cells. GM-CSF[227], G-CSF[158] , SDF-1[228], hepatocyte growth factor (HGF)[229] and NO[230] all stimulate HPC growth, differentiation and migration in vivo and in-vitro. It has been proposed that the direct cell-to-cell contact may be a stimulus of progenitor cell differentiation. Studies show that direct cell-to-cell contact between MSCs and cardiomyocytes or smooth muscle cells results in MSC differentiation into these two cell types[180]. Co-culture of MSCs with HPCs drives MSCs to differentiate into endothelial-like cells[130]. Shear stress, the mechanical force generated by blood flow, can also effectively induce expression of endothelial-specific genes in stem cells [246, 247]. Laminar flow can enrich both adult and embryonic stem cell populations for endothelial progenitors [246, 247].

1.9 The case for further clinical studies

The vast majority of studies on the role of vascular progenitors following arterial injury have been carried out in animal models. Human studies have mainly focused

on patients undergoing coronary angioplasty[100, 154]. It has been shown that the contribution of bone marrow derived progenitor cells to the arterial wall may vary depending on the type of injury[95]. Transplantation of bone marrow cells from transgenic mice that globally express GFP into a wild type mice, reveals that three distinct types of mechanical injury produce varying degrees of bone marrow derived cell contribution to the arterial wall. In the first injury model, a 0.38 mm straight spring wire was inserted into the mouse femoral artery to denude and dilate the artery. This model best recapitulates endarterectomy procedures in humans, since it involves both vessel wall dilatation and denudation of the vessel intima and media. In the second model, a polyethylene tube was placed around the mouse femoral artery (perivascular cuff induced injury). In the last model, the mouse common carotid artery was ligated just proximal to the bifurcation. Wire injury led to large numbers of GFP^+ cells in both the media and the neointima, whereas perivascular cuff placement and carotid artery ligation resulted in significantly fewer GFP^+ medial and neointimal cells. The authors also studied the fate of the bone marrow derived cells by examining α-SMA expression. Whereas a significant number of GFP^+ cells in the neointima and the media after wire injury were also $\alpha\text{-SMA}^+$, only a few of the GFP^+ cells expressed α-SMA in the other two injury models. It is possible, therefore, that the response to CEA, which involves not only loss of endothelium, but also stripping of the media, may differ in the magnitude and character of progenitor cell involvement when compared to coronary angioplasty. The amount of injury caused by CEA may also have an impact on the effects of locally derived tissue progenitors, which may indirectly

affect the need for more distant blood borne progenitors. It is also known that carotid atheroma appears later in life than coronary plaques [248], this may reflect a mechanical difference or differences in the pathogenesis of atherosclerosis between small and large vessels. The shear stress in the coronary arteries is also different to that in the common carotid artery. These factors may all contribute to differences in the response to arterial injury between coronary and carotid arteries.

1.9.1 Carotid endarterectomy as a clinical model

The risk of restenosis after carotid endarterectomy is around 10% - 20% [29-31]. This is difficult to define precisely because of the differing definitions and different duplex criteria used to assess this. Restenosis following carotid endarterectomy carries a low risk of stroke (1-10%) [30, 31]. A recent systematic review showed that a second intervention (stenting or surgery) for restenosis occurred in ~3% of all treated arteries [249]. This is comparable with the results of ECST, where <1% (5/558) of the surgical patients had a second CEA because of a symptomatic restenosis. It is evident from these studies that patients with restenosis after CEA generally follow a benign course. Therefore the potential direct clinical impact of future therapies designed to reduce the risk of restenosis is small, and this intern raises questions as to the value of studying restenosis in the carotid artery.

We feel that carotid endarterectomy as a model has a number of advantages. The low re-intervention rate allows us to study the impact of an isolated arterial injury on HPC number and function and the subsequent degree of restenosis. As a

relatively superficial artery, we can assess the degree of restenosis using duplex ultrasound, which is accurate and safe for the patient. Furthermore the results of this study will give a mechanistic insight into restenosis and response to arterial injury and will therefore be applicable to all arteries not just to the carotid.

In trying to study arterial injury and restenosis we considered and rejected other potential human models. Percutaneous lower limb angioplasty was rejected as it was felt that the type of injury, and therefore the progenitor cell response, might be similar to that of angioplasty in the coronary, the main difference being anatomical location. Patients with peripheral vascular disease also have multiple sites of stenosis and can have patency rates as low as 50% at 1yr [250] and therefore a high re-intervention rate. Preliminary investigations revealed that in our institution a very low number of patients have a primary angioplasty for an isolated lesion. This would make it difficult to study the progenitor cell response to an isolated injury and correlate this with restenosis without the confounding factor of previous or future angioplasties. Femoral endarterectomy as an alternative model was rejected due to low numbers and also as the procedure is often carried out in association with simultaneous or previous angioplasty.

1.10 Hypothesis and Aims

Blood borne vascular progenitor cells are essential components of the repair mechanism following arterial injury. Mobilisation and/or activity of these cells will influence re-endothelialisation and the generation of neointimal hyperplasia and thus restenosis following carotid endarterectomy.

We aimed to address this hypothesis by:

1. Carrying out a longitudinal study in which the HPC and MSC numbers and activity would be measured before and after carotid endarterectomy and correlating their numbers and function with the degree of restenosis

2. Measuring the expression of factors that are known to mobilise these progenitor cells i.e VEGF, SDF-1, GM-CSF and PlGF

2. METHODS AND STUDY DESIGN

2.1 Measurement of HPC number by flow cytometry

2.1.1 Overview of investigations

Whole blood was labelled using fluorescently conjugated monoclonal antibodies against CD34-peridinin chlorophyll protein (PerCP, Becton Dickinson, BD), VEGFR2-phycoerythrin (PE, R&D Systems) and CD133-PE (Miltenyi). Whole blood was also separately labelled with CD133 primary (Miltenyi) and a fluorescently conjugated secondary (goat anti-mouse fluorescein isothiocyanate (FITC) - Dako). Flow cytometry was carried out on a FACScan flow cytometer (BD) to identify and enumerate HPC numbers ($CD34^+/CD133^+$/VEGFR2).

2.1.2 General methods

2.1.2.1 Direct Immunofluorescence staining of whole blood.

1. 500µl of whole blood was aliquoted into an Eppendorf tube.
2. 20µl of FcR block (Miltenyi) was added and incubated for 20 mins.
3. A given concentration of antibody (Table 4) was added, mixed well and incubated in the dark at 4ºC for 30 mins. Non-immune IgG, of the same isotype as the test antibody, raised in the same animal, and conjugated to the same fluorescent probe was added to a duplicate sample of the same blood at the same concentration as the test antibody to act as a control.

4. 5mls of Pharmalyse (BD) was added to the tube, mixed well and incubated in the dark at room temperature for 10 mins.
5. The tube was centrifuged at 400g for 5 mins and the supernatant discarded.
6. The cells were resuspended in 5mls of stain buffer (Dulbecco's modified phosphate buffered saline, Ph7.4 [DPBS], 0.09% sodium azide, 2% heat inactivated foetal calf serum, FCS).
7. Wash steps 5 and 6 were repeated for a total of three washes.
8. The pellet was resuspended in 2mls of stain buffer.
9. Data was acquired by flow cytometry.

2.1.2.2 Indirect Immunofluorescence staining of whole blood.

1. 500µl of whole blood was aliquoted into an Eppendorf tube.
2. AS STEP 3 IN SECTION 2.1.2.1.
3. The blood was washed by adding 5mls of cold stain buffer, mixed and centrifuged at 400g for 5 minutes. The resulting supernatant was then discarded, and the pellet resuspended in 5mls stain buffer.
4. Step 3 was repeated three times.
5. The pellet was resuspended in 500µl of PBS containing 5% serum from the animal the secondary antibody to be used was raised in, and the mixture incubated for 10 mins.
6. Fluorescently labelled secondary antibody was added to both test and control tubes, mixed well and incubated in the dark at 4ºC for 30 mins.

7. 5mls of Pharmalyse (BD) was added to the tube, mixed well and incubated in the dark at room temperature for a further 10 mins.
8. The tube was centrifuged at 400g for 5 mins and the supernatant discarded.
9. The cells were resuspended in 5mls of stain buffer (section 2.1.2.1).
10. Wash steps 8 and 9 were repeated for a total of three washes.
11. The pellet was resuspended in 2mls of stain buffer. If co-staining with directly labelled antibodies, the pellet was resuspended in 500ul of stain buffer and the protocol for direct immunofluorescent staining (section 2.1.2.1) was followed from step 2.
12. Data was acquired by flow cytometry.

2.1.2.3 Cell fixation for flow cytometry

1. The direct immunoflourescence staining protocol (2.1.2.1) was followed up to and including step 7.
2. The pellet was resuspended in 5mls 5% formaldehyde in stain buffer.
3. The sample was incubated at room temperature for 5mins.
4. The tube was centrifuged at 400g for 5mins and the supernatant discarded.
5. The cells were resuspended in 5mls of stain buffer and steps 4 and 5 were repeated for a total of 3 washes.
6. The pellet was resuspended in 2mls of stain buffer and stored at 4ºC overnight.

2.1.2.4 Design of optimization experiments

It was initially necessary to determine whether individual antibodies detected specific cell populations. Blood samples were therefore stained with individual antibodies whose binding was then optimised in the following ways.

- Comparing different antibody concentrations (Table 4).
- Comparing incubation temperatures - 4ºC and room temperature.
- Comparing incubation times - 10, 20 and 30 mins.

Table 4. Concentrations of antibodies used for optimization experiments.

Antibody	**Antibody concentration (**µg/ml)			
CD34-PerCP	0.125	0.25	0.5	0.75
CD133-PE	0.03	0.17	0.33	0.50
VEFR2-PE	0.125	0.25	0.50	0.75
CD133 primary	0.05	0.10	0.50	1.00

Both CD133 and VEGFR2 primary antibodies were both directly conjugated to PE (Table 4) and could therefore not be used together in a double or triple stain. Neither of these antibodies was available directly conjugated to the alternative fluorescent probe FITC. The mouse anti-human CD133 primary was therefore detected using a goat anti-mouse FITC conjugated secondary antibody (200µg/ml), with the indirect immunofluorescence staining protocol. Triple positive cells were

then stained together with CD34-PerCP and VEGFR2-PE. This staining protocol was further optimised by investigating the following.

1. Minimising non-specific binding in the triple stain

Reduction of non-specific binding was carried out by including a blocking step (step 2 direct immunofluorescent staining protocol 2.1.2.1). The following blockers were investigated.

i) FcR block (BD, UK)

ii) 5% goat serum (Invitrogen, UK)

iii) Mouse immunoglobulin (IgG, Invitrogen, UK, 0.5 µg/ml)

iv) 5% bovine serum albumin(Invitrogen, UK)

2. Using a mixed isotype control to check for stain specificity.

Two methods of controlling for co-expression of the three antigens were assessed.

i) Conventional control, where all isotype IgG controls were added to a duplicate blood sample.

ii) Mixed control in which each primary antibody was replaced, in turn, by its isotype control i.e substituting CD133-FITC for it's isotype control (IgG-FITC, VEGFR2-PE, CD34-PerCP); or CD34-PerCP with it's isotype control (CD133-FITC, VEGFR2-PE, IgG-PerCP).

3. Assessing the effect of formaldehyde fixation of the sample after staining, storing the stained sample at 4^{o}C and analysing the sample the next day (16 hrs).

4. Determining the intra-assay variability.

This was done by measuring cell numbers in 6 replicates of the same blood sample processed on the same day.

2.1.3 Results

2.1.3.1 The effect of varying antibody concentration, incubation time and temperature on the detection of single positive cells.

The effect of varying antibody concentration on the detection of single positive cells using the direct immunostaining protocol is shown in Figs 9-12. Optimal staining was achieved when using a concentration of 0.5µg/ml of CD34 PerCP and 0.33µg/ml of CD133-PE. Higher antibody concentrations increase the non-specific binding (binding of isotype control) and as a result no overall increase in the number of positive cells was detected. The optimal concentration was less clear with the VEGFR2-PE antibody (Fig 11). Concentrations greater than 0.5µg/ml showed no overall increase in the number of positive cells. However at this concentration the number of positive cells detected in the isotype control was very high (~200) giving a false positive rate of 36%. A concentration of 0.125µg/ml detected fewer overall positive cells, but also had a lower false positive rate of 17%. Optimal staining for the CD133 primary antibody was achieved at a concentration of 0.5µg/ml using the indirect immunofluorescence staining protocol (Fig 12).

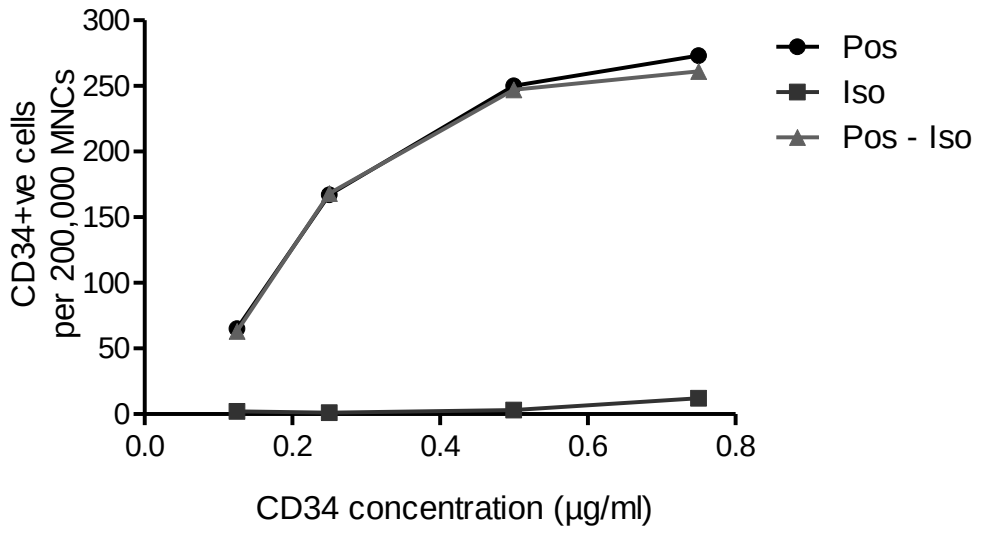


Fig 9. The effect of varying CD34 - PerCP antibody concentration (Pos), with its isotype equivalent (Iso) on the number of single positive cells (Pos-Iso) detected by flow cytometry. (n=3 at each concentration)

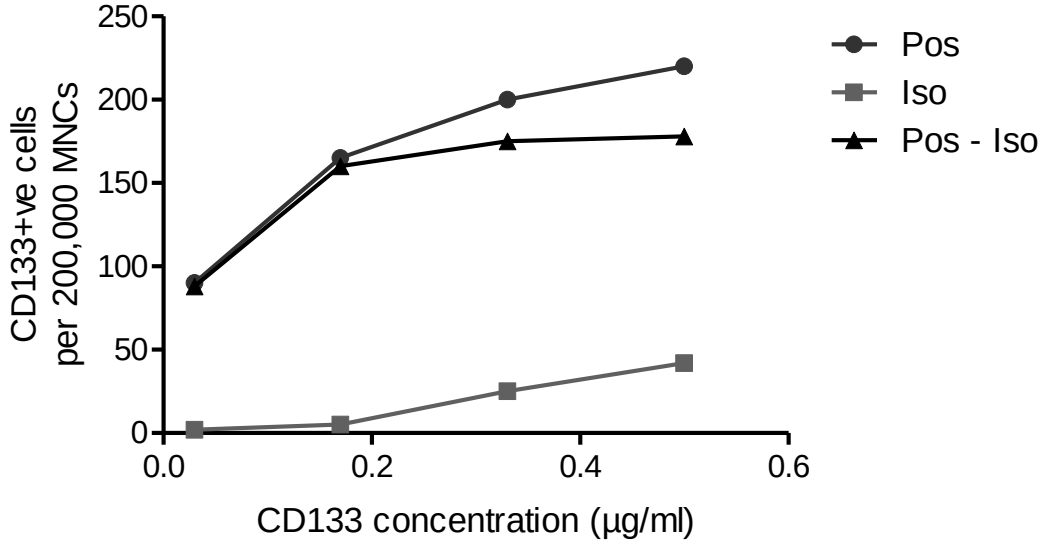


Fig 10. The effect of varying CD133 – PE (Pos) antibody concentration, with its isotype equivalent (Iso) on the number of overall single positive cells (Pos-Iso) detected by flow cytometry.

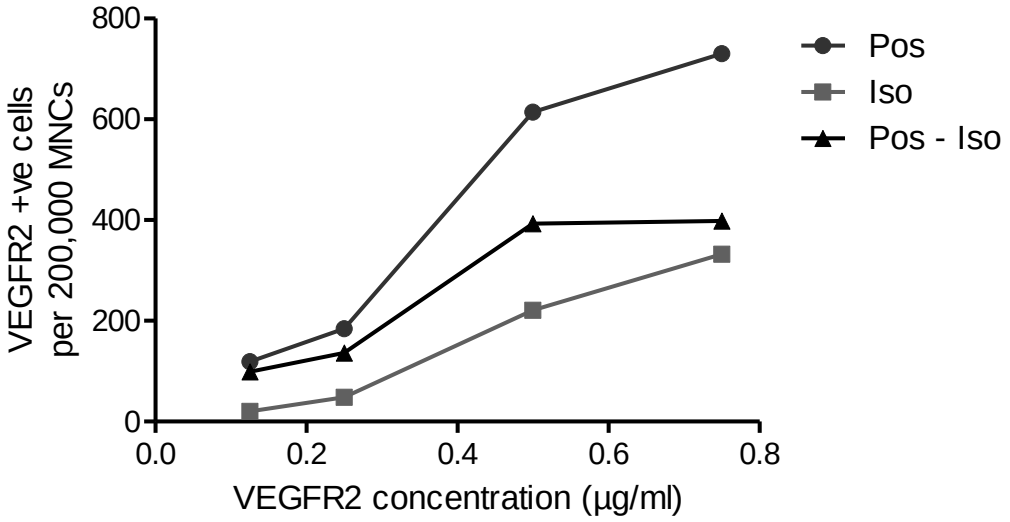


Fig 11. The effect of varying VEGFR2-PE (Pos) antibody concentration, with its isotype equivalent (Iso) on the number of overall single positive cells (Pos-Iso) detected by flow cytometry.

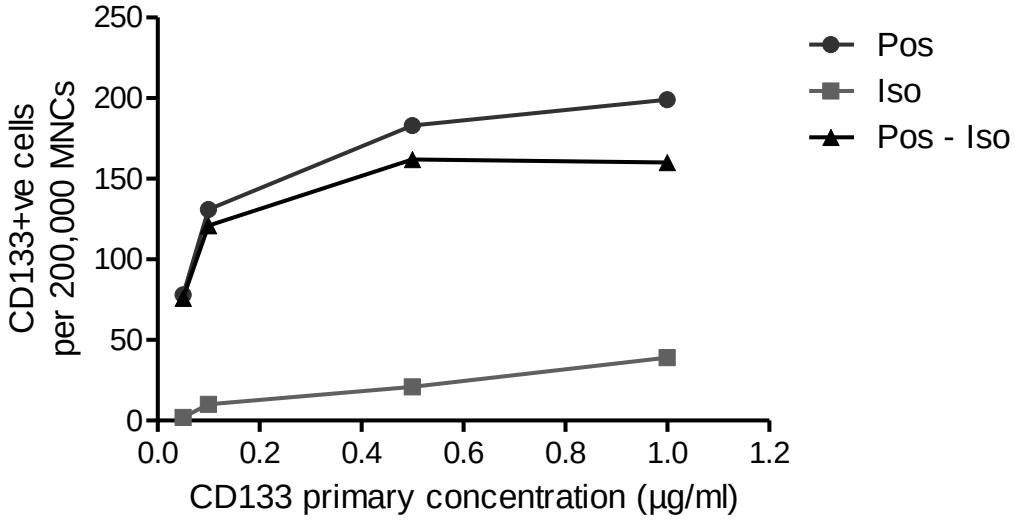


Fig 12. The effect of varying CD133 primary antibody concentration, with its isotype equivalent (Iso) on the number of overall single positive cells (Pos-Iso) detected by flow cytometry (after staining with FITC- goat anti-mouse secondary).

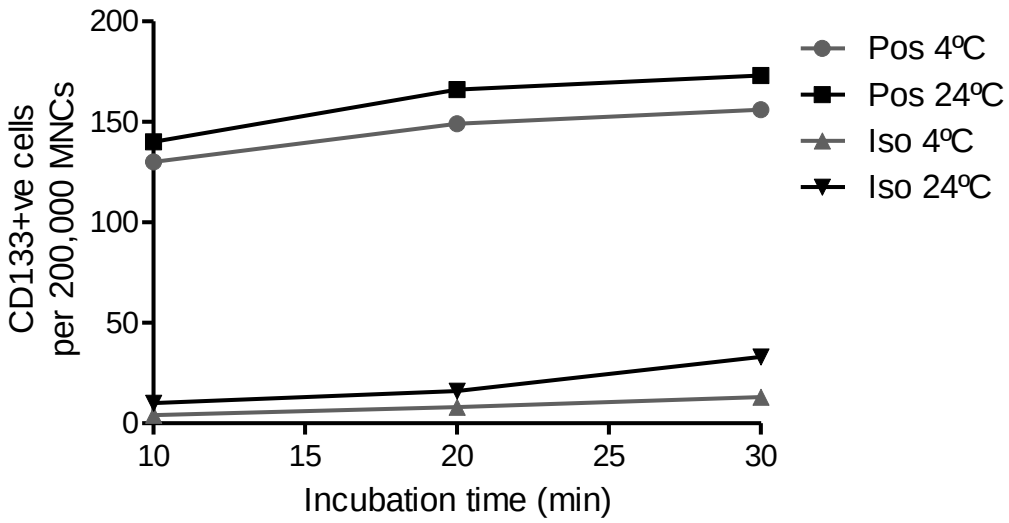


Fig 13. The effect of varying incubation time and temperature on detection of single positive cells.

Increasing incubation time from 10 to 30 minutes increased the number of CD133 +ve cells detected (CD133-PE, Fig 13). However, when incubated at room temperature there was also a rise in the number of false positives detected by the isotype control. This did not occur when the incubation was carried out at 4ºC. The optimal conditions were incubation at 4ºC for 30 minutes.

2.1.3.2 Detection of Triple Positive Cells

Whole blood was triple stained for CD133 using the IgG-FITC secondary antibody, indirect immunofluorescence protocol. Cells were then stained for VERGFR2-PE and CD34 PerCP using the direct immunofluorescence protocol. The gating system used for detecting triple positive cells is shown in Figs 14a-d. Previous work has shown that the progenitor cell pool lies within the mononuclear cell region

(lymphocyte and monocyte). In addition the degree of non-specific binding of the fluorescent antibodies within the granulocyte region obscures the signal from the mononuclear region. Therefore only the mononuclear region is analysed further.

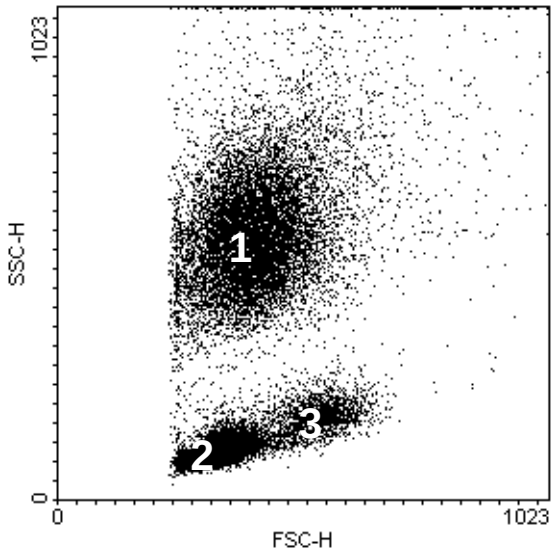


Fig 14a. Plot of forward scatter (FSC) versus side scatter (SSC) from flow cytometry of human blood . Area **1** represents granulocytes, **2** lymphocytes and **3** monocytes. Only cells in area 2 and 3 were further analysed.

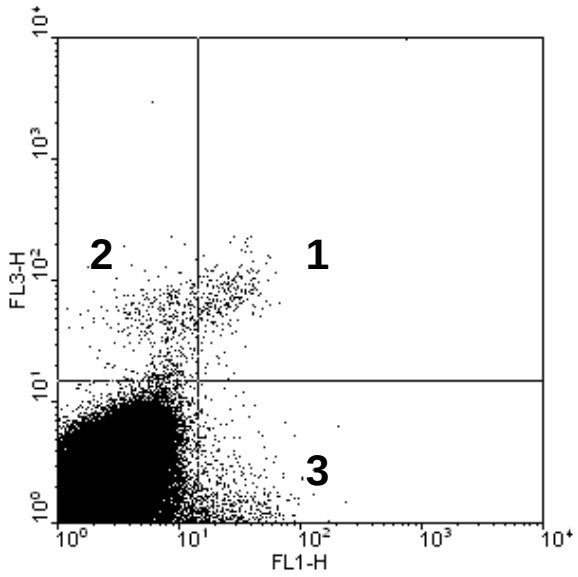


Fig 14b. Dot plot of CD133 FITC (FL1) versus CD34-PerCP (FL3). Quadrant **1** contains cells that are double positive for CD133 and CD34; **2** cells that are CD34 positive only; and **3** cells that are CD133 positive only.

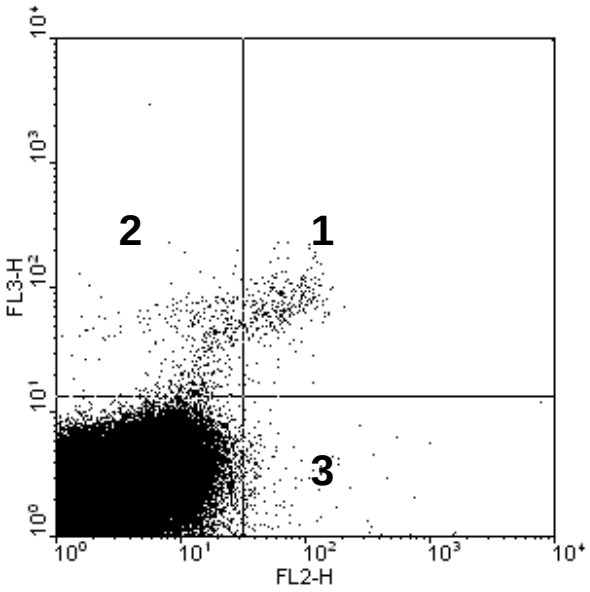


Fig 14c. Dot plot of VEGFR2 - PE (FL2) versus CD34-PerCP (FL3). Quadrant **1** contains cells that are double positive for VEGFR2 and CD34; **2** cells that are CD34 positive only; and **3** cells that are VEGFR2 positive only.

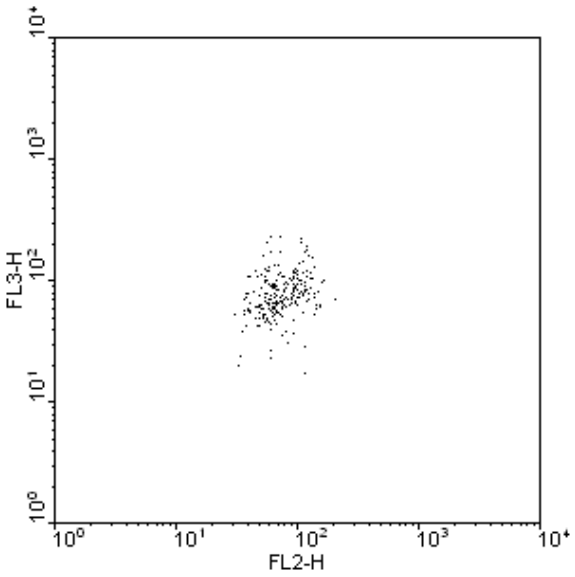


Fig 14d. Dot plot of VEGFR2 - PE (FL2) versus CD34-PerCP (FL3).

Only cells present in quadrant 1 from Figs 14b and c were denoted as triple positive (CD133, VEGFR2 and CD34) cells.

2.1.3.3 Comparison of conventional and mixed isotype controls

Conventional isotype controls were compared with mixed isotype controls to determine the specificity of the triple stain (Table 5). Use of a conventional isotype control (i.e three fluorescently labelled isotype antibodies in an identical tube) revealed 16 triple positive cells compared with 119 triple positive cells visualised by the marker antibodies, giving a false positive rate of 13%. Analysis using a mixed isotype control gave a false triple positive rate of 14% and 16% for CD133 and CD34 IgG isotypes respectively. Substituting VEGFR2-PE for it's isotype control (i.e CD133-FITC, IgG-PE, CD34-PerCP) resulted in a very high false positive rate of 60% (Table 5).

Table 5. Mean (n=3) of the numbers of fluorescent cells stained using conventional and mixed isotype controls

FITC antibody	PE antibody	PerCP antibody	Triple positives (±SEM)	False positive rate (%)
CD133-FITC	VEGFR2-PE	CD34-PerCP	119(±7)	N/A
IgG-FITC	IgG-PE	IgG-PerCP	16(±2)	13
IgG-FITC	VEGFR2-PE	CD34-PerCP	17(±3)	14
CD133-FITC	VEGFR2-PE	IgG-PerCP	19(±2)	16
CD133-FITC,	IgG-PE	CD34-PerCP	72(±5)	60

This experiment was repeated on eight healthy volunteers with similar results (Fig 15). The mixed isotype control for the VEGFR2-PE (i.e CD133-FITC, IgG-PE, CD34-PerCP) showed consistently high numbers of false positives, compared with the normal isotype controls (P=0.001, paired t-test), although consistently and significantly lower than the triple primary antibody-treated sample (P=0.001, paired t-test).

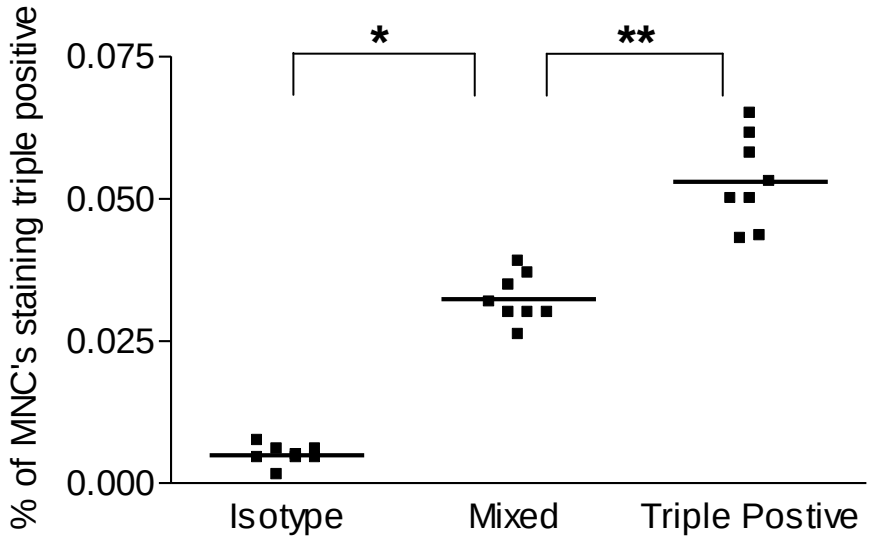


Fig 15. The percentage of MNCs detected following staining for conventional isotype control, mixed isotype control and CD133^{+ve}/CD34^{+ve}/VEGFR2^{+ve}. (n=8, P<0.001 for both mixed vs isotype* and vs triple, paired t-test)**

In order to determine whether the high degree of false positives was only a problem related to theVEGFR2 antibody from R&D Systems, these experiments were repeated using anti-VEGFR2-PE and matched isotype antibodies obtained from Miltenyi, UK and BD, UK. A triple stain alongside a mixed isotype control found these antibodies to have a false positive rate of 54% and 59% respectively.

2.1.3.4 Blocking non-specific binding in CD133/CD34/VEGFR2 triple stain

Various blocking methods were investigated in order to reduce the number of false positives in the triple stain (2.1.2.4). Blocking with goat serum gave the lowest level of false positives (42%, false positive rate was calculated as a percentage of the triple positive stain – 155(±5), Table 6), but none of the blocking reagents resulted

in marked fall in the number of false positives compared with a mixed control**.** A double staining technique (co-expression of CD133 and CD34) was therefore adopted to define HPC's in this study as both isotype and mixed controls had low number of false positives (~15%) using these antibodies (Table 5).

Table 6. Comparison of blocking protocols in assessing mixed isotype controls staining.

	Mean cell numbers (n=3)	SEM	% false positive
Conventional isotype Control	25	3	16
FcR block mixed isotype	71	4	45
Goat Serum mixed isotype	65	5	42
Mouse IgG mixed isotype	78	8	50
BSA mixed isotype	76	5	49
Mixed isotype (no block)	80	3	52

2.1.3.5 Optimisation of CD133/CD34 double labeling

i) FcR blocking The utility of the FcR blocking reagent was investigated by incubating paired blood samples from 5 volunteers in either 20µL of FcR reagent for 10 minutes followed by the primary antibody, or directly with the antibody (direct immunofluorescence staining protocol 2.1.2.1). FcR blocking had no significant effect on the number of double positive cells detected (Fig 16, P=0.68, Wilcoxon signed ranks test)

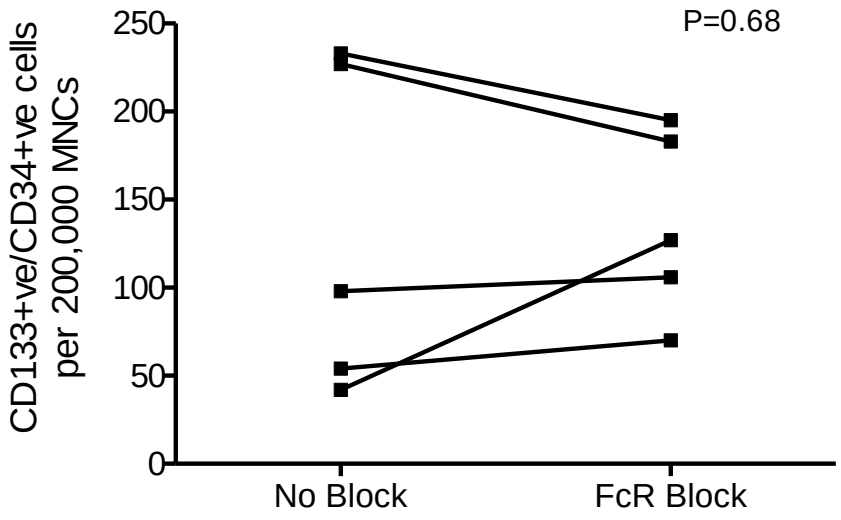


Fig 16. Effect of FcR blocking on CD133+ve/CD34+ve analysis (n=5, Wilcoxon signed ranks test)

ii) Cell fixation Paired blood samples from five volunteers were either processed for immediate flow cytometry (CD34/CD133 staining) or fixed in 2% formaldehyde and analysed 16 hours later (cell fixation protocol 2.1.2.3). There

was no significant difference between the cell numbers detected following each treatment (Fig 17, P=0.68, Wilcoxon signed ranks test).

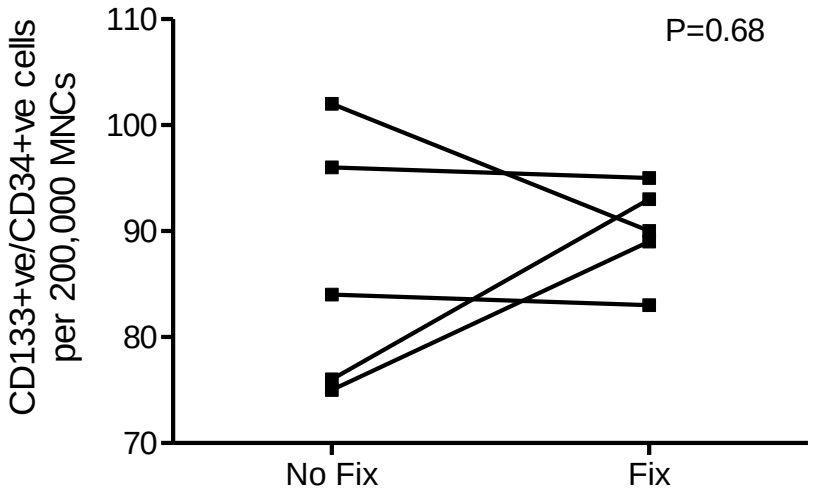


Fig 17. Effect of fixation on CD133+ve/CD34+ve analysis (n=5, Wilcoxon signed ranks test)

2.2.3.6 Intra-assay variability of double staining

Six blood samples from the same volunteer were stained with CD133-PE and CD34-PerCP. A duplicate set of 6 blood samples from the same volunteer was stained with isotype controls (IgG-PE and IgG-PerCP). Assay variability for the detection of double positive cells was 6.3%. (Table 7)

Table 7. Intra-assay variability of flow cytometric analysis of HPC number using CD133+ve/CD34+ve staining

Antibody / IgG control used	Mean(SD) n=6	Variability
CD133-PE + CD34-PerCP	150.3(±9.5)	6.3%
IgG-PE + IgG-PerCP (isotype double)	3 (±1.2)	40%

2.1.4 Discussion

There is no consensus as to the most specific markers of an HPC as measured by flow cytometry. Most have defined these cells by the co-expression of both haematopoietic (CD34/CD133) and endothelial (VEGFR2) marker proteins. Detection of HPCs using a triple stain (VEGFR2/CD133/CD34) and a conventional isotype control (isotype IgGs in a separate tube), shows that approximately 0.05% of the circulating PBMNC pool is comprised of HPCs, with a low number of false positives (~8%). Our results with a mixed control for CD133/CD34/VEGFR2 staining (substitution of the VEGFR2 antibody for its isotype control IgG in the reaction mix) suggest, however, that use of the conventional isotype control may grossly overestimate the number of HPCs in blood, as mixed control staining revealed a high number of false positives (~60%) caused by the non-specific binding of the VEGFR2-PE antibody. This problem was encountered with VEGFR2

antibodies from 3 manufacturers (R&D Systems, BD and Miltenyi). The high level of false positives may of course be the result of the staining protocols used, but I was unable to reduce the number of these false positives with a variety of protocols. Double staining using CD34+/CD133+ was optimised and adopted for all subsequent analysis in this study; a combination that has also been adopted by others[251-253].

2.1.5 Conclusions

- The use of conventional isotype controls alone may overestimate the number of circulating HPCs.
- The measurement HPCs numbers by flow cytometry may be confounded by the source of the VEGFR2 antibody.
- Optimal double staining was achieved by incubating 0.5µg/ml of CD34 PerCP and 0.33µg/ml of CD133-PE for 30 min at 4°C.
- FcR block does not increase the specificity of staining and was therefore not used.
- Cell fixation and analysis up to 16 hours later is accurate.
- The double stain technique is precise, with an intra-assay variability of 6.3%.

2.2 HPC culture and colony forming unit (CFU) assay

2.2.1 Overview of investigations

Peripheral blood mononuclear cells were isolated by Ficoll gradient density centrifugation and placed on fibronectin-coated plates in EGM-2 culture medium as previously described for endothelial progenitor cell analysis (EPC)[111]. The number of colonies was counted 5days after culture under phase contrast microscopy. The cultured cells were stained with Dil-acLDL (Molecular Probes) and ULEX Lectin (Sigma) and the endothelial markers, CD31 (Dako) and VEGFR2 (Dako), with only double positive cells regarded as endothelial cells. In order to grow OECs/late HPCs a separate culture was continued for up to 4 weeks, with media change every other day as previously described[104].

2.2.2 General methods

2.2.2.1 Peripheral Blood Mononuclear Cell (PBMNC) Isolation

1. Blood was transferred to a sterile 50ml falcon tube and diluted with pre-warmed Dulbecco's modified eagle medium (DMEM) to two times the original volume.
2. 50ml Falcon tubes were filled with 20ml Ficoll-Paque Plus (GE Healthcare).

3. 25ml of the diluted blood suspension was gently layered on top of the Ficoll-Paque Plus.
4. The layered solution was centrifuged at 840g for 25 mins with the brake on the centrifuge switched off.
5. The PBMNC fraction is clearly seen as an opaque ring of cells (the first from the top of the tube, Fig 18). This was carefully removed using a sterile pasteur pipette, and transferred to a fresh 50 ml tube.
6. The cells were washed by addition of pre-warmed DMEM making a final volume of 40ml. The cells were centrifuged at 400g for 5 mins.
7. The supernatant was discarded and the cells resuspended and washed as described in step 6.
8. The cell pellet was resuspended in EGM-2 supplemented with the Bullet Kit system (Clonetics) and 5% FCS (Sigma) [called EBM-2] to a volume one quarter the original volume of blood.
9. Cell numbers were counted using a haematocytometer and adjusted to 2.5×10^6 cells per ml.

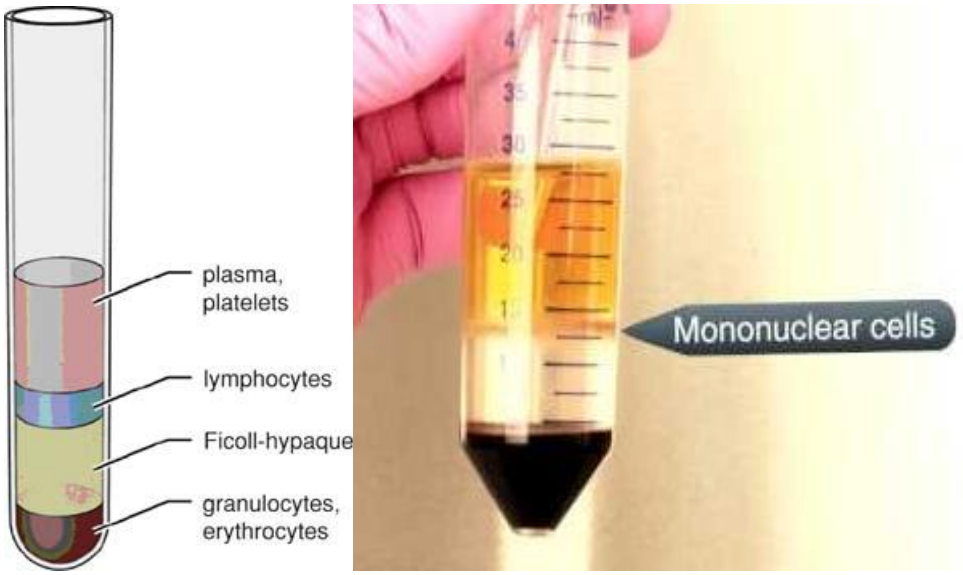


Fig 18. Ficoll gradient isolation of mononuclear cells

2.2.2.2 Haematopoietic progenitor cell culture

1. Six-well plates were pre-coated with 1ml of fibronectin solution (10μg/ml in M199 culture medium - Sigma, UK) per well and incubated overnight at 4ºC. Chamber slides (Nunc, UK) were also similarly treated by coating with 0.5mls fibronectin solution.
2. Any excess fibronectin solution was aspirated and the plates placed in an incubator (37°C) for 10 mins.
3. 4mls (1 x10^7 cells per well) of the mononuclear cell suspension (step 9 section 2.2.2.1) was pipetted into each well. Alternatively 2mls of the cell suspension was added to each chamber of the fibronectin pre-coated chamber slides.
4. The cells were incubated in 5% CO_2 at 37°C for 48hrs.

5. The non-adherent fraction was removed at 48hrs by gently aspirating the fluid and replaced with 2mls pre-warmed EBM-2.
6. Half the volume of medium was changed every 48hrs thereafter.
7. HPC colony forming units (eCFU) were counted at day 5 under phase contrast microscopy.
8. Cell culture was continued in order to grow OECs/late HPCs with media change every 48hrs for up to 4 weeks.

2.2.2.3 Dil-acLDL and lectin staining

1. After 5 days of culture, HPCs grown on chamber slides (Nunc) were washed twice with 1 ml warmed sterile phosphate buffered saline.
2. The cells were incubated with 5µg/ml of Dil-acLDL (Molecular Probes) in warmed EGM-2 (Cambrex) at 37°C for 1 hour.
3. The cells were washed twice before fixation with 2% paraformaldehyde in PBS for ten minutes.
4. The cells were washed twice before incubation with Lectin from Ulex europaeus agglutinin (Lectin UEA-1, Sigma) – 10µg/ml in PBS for 1hr in the dark at room temperature.
5. The slides were washed twice with PBS and mounted in Vectashield (mounting medium containing the blue fluorescent nuclear stain, DAPI, Vector Laboratories).
6. The slides were viewed under a fluorescent microscope.

2.2.2.4 Immunohistochemical staining with CD31 and VEGFR2

1. HPCs grown on chamber slides for 5 days were washed twice with 1ml warmed sterile PBS before being fixed with 2% paraformaldehyde in PBS for ten minutes.
2. The slide was washed twice with PBS.
3. Primary antibody(s) and control(s) were made to the required dilutions (CD31(Dako) 1 in 40, VEGFR2(Dako) 1 in 200 - in PBS containing 5% normal serum from the same species the secondary antibody was raised in.
4. 1ml of this solution was added to each chamber slide. Control isotype IgGs at the same concentrations as primary antibody were added to a duplicate chamber.
5. The slide was incubated for 30 mins at room temperature and washed twice with PBS
6. Fluorescently labelled secondary antibody(s) were made to the recommended dilution(Goat anti-rabbit (Dako)1:750, goat anti-mouse (Dako,) 1:100) in PBS with 5% serum from the animal the secondary was raised in,
7. 1 ml of the solution was added to the chamber slides and incubated in the dark at room temperature for 30 minutes.
8. The slides were washed twice with PBS and mounted in Vectashield
9. The slides were viewed under a fluorescent microscope.

2.2.2.5 Optimisation experiments for HPC-CFU growth

The following experiments were carried out in order to optimise the CFU assay.

- *Assessing different batches and sources of FCS*. Foetal calf serum obtained from Sigma, UK – 3 batches; Hyclone, UK, 3 batches; and Gibco, UK, 2 batches were assessed at varying the concentration in the EBM-2. (20%, 10%, 5%)
- Batch testing the fibronectin used to pre-coat the wells (from Sigma,Gibco, BD)
- Pre-plating the PBMNC. This involved incubating the non-adherent fraction at 48hrs in 12-well plates. At step 5 (section 2.2.2.2), wells were vigorously pipetted and the non-adherent cells were collected, washed with warmed PBS before being re-suspended in EBM-2. Cells were plated at a concentration of 5×10^6 cells per well in a fibronectin-coated 12 well plates.
- Assessing the effect of changing the plating concentration of PBMNCs in step 3 of 2.2.2.2 (HPC CFU culture, $5x10^6$, $2.5x10^6$, $1.25x10^6$, $6x10^5$ cells/ml).
- Assessing the intra-assay variability and inter-observer error of the eCFU assay.

2.2.3 Results

2.2.3.1 HPC-eCFU assay

MNCs from healthy volunteers were isolated and eCFU cultures commenced (2.2.2.2) and stained for endothelial markers (2.2.2.3 and 2.2.2.4) on day 1 and

day 5. HPC cultures at day 1 did not stain for Dil-acLDL, Lectin, CD31 or VEGFR2. By day 5 cells had the typical appearance of spindle shaped endothelial cells (Fig19a) and stained double positive for CD31 and VEGFR2 (Fig19b), and for Dil-acLDL and Lectin (Fig19c), which suggests an endothelial phenotype.

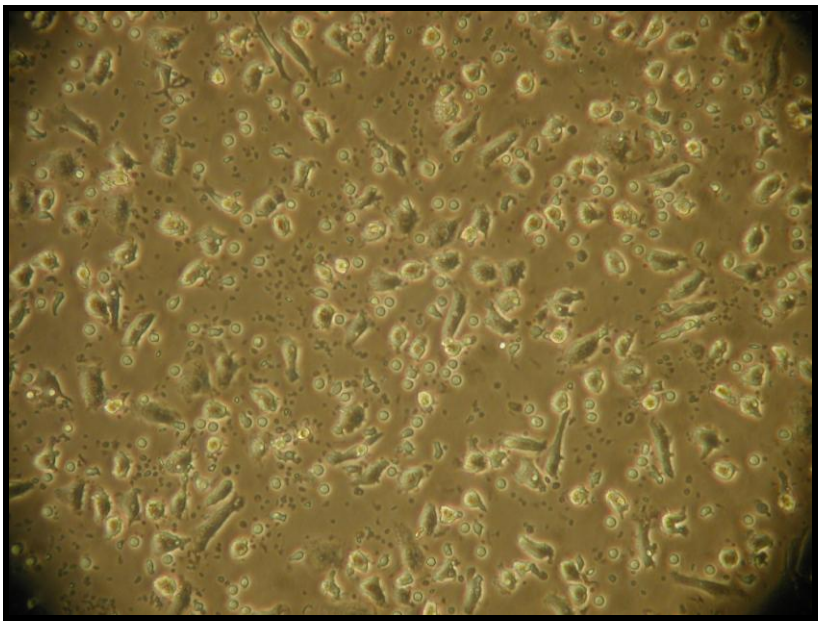


Fig 19a. Light microscope (phase contrast) picture of HPCs (day 5, x 20 mag).

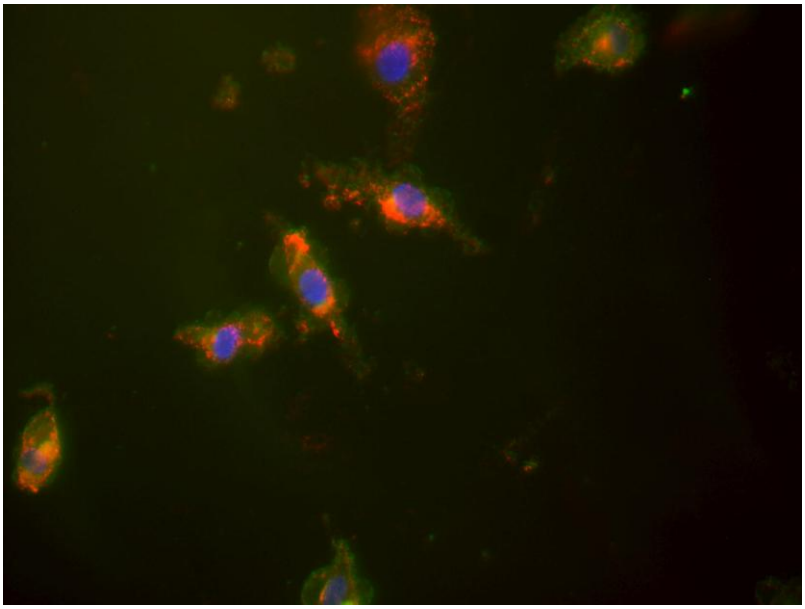


Fig 19b. Day 5 HPC culture stained with VEGFR2 (green) and CD31(red). Yellow orange fluorescence also shows dual stained cells. Blue fluorescence denotes nuclear stain. (x40 mag)

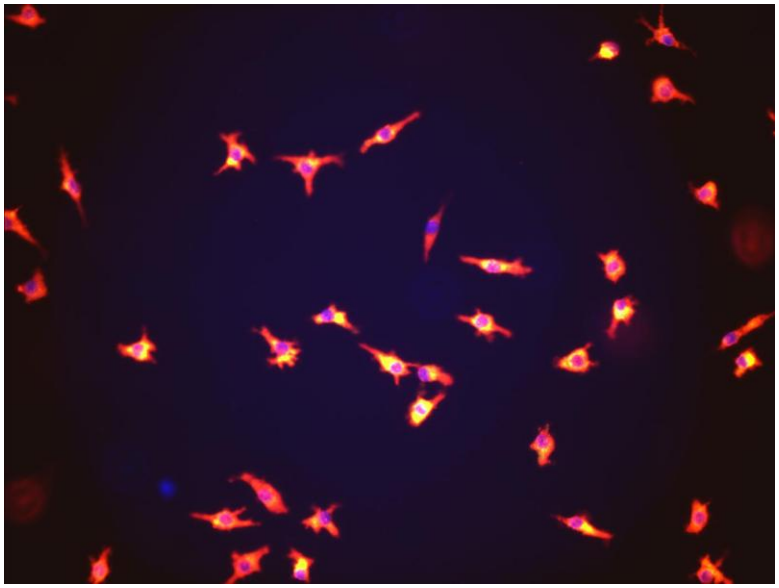


Fig 19c. Day 5 HPCs stained using Dil-acLDL(red) and lectin-FITC (green/yellow) . Blue fluorescence denotes nuclear stain. (x 20 mag)

Batch testing of FCS and fibronectin from different companies showed that there were important differences between sources of these reagents in terms of success rate of the eCFU assay. Colony growth only occurred in the presence of a specific batch of FCS and fibronectin obtained from Sigma (Fig 20a). Other batches of FCS and fibronectin did not yield eCFUs though most supported the growth of spindle shaped cells. Colonies began to grow at day 3 and were clearly recognisable by day 5 (Fig 20a and 22) after which time they began to disperse.

Pre-plating the PBMNCs and culturing the non adherent fraction after 48 hrs as has been previously described[111] did not result in eCFU formation. In addition CFUs did not appear when cell concentrations were below 2.5 $x10^6$ cells/ml ($1x10^7$ per well, Table 8). Changing the culture media with fresh EBM-2 more frequently than every 48 hrs had no effect on the formation of CFU's.

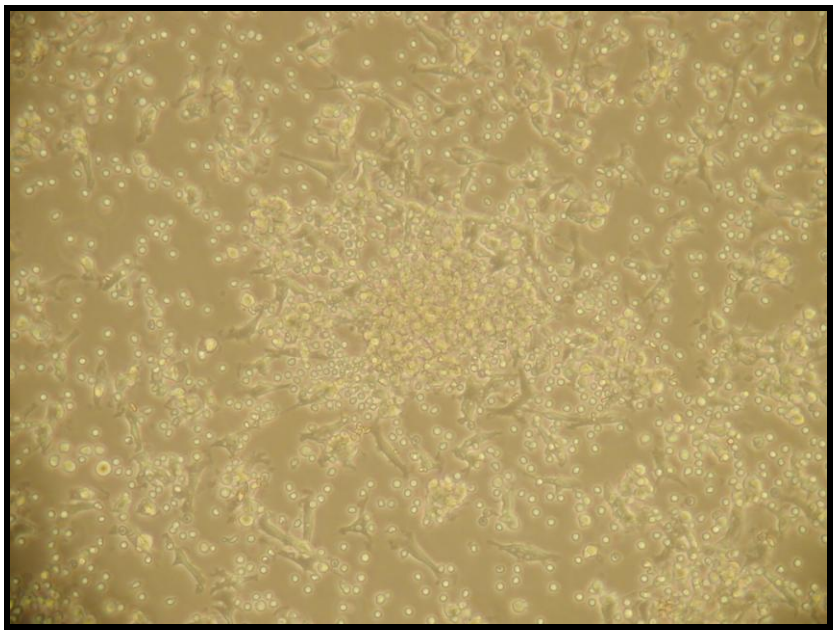


Fig 20a. A typical CFU after 6 days in culture (magx10).

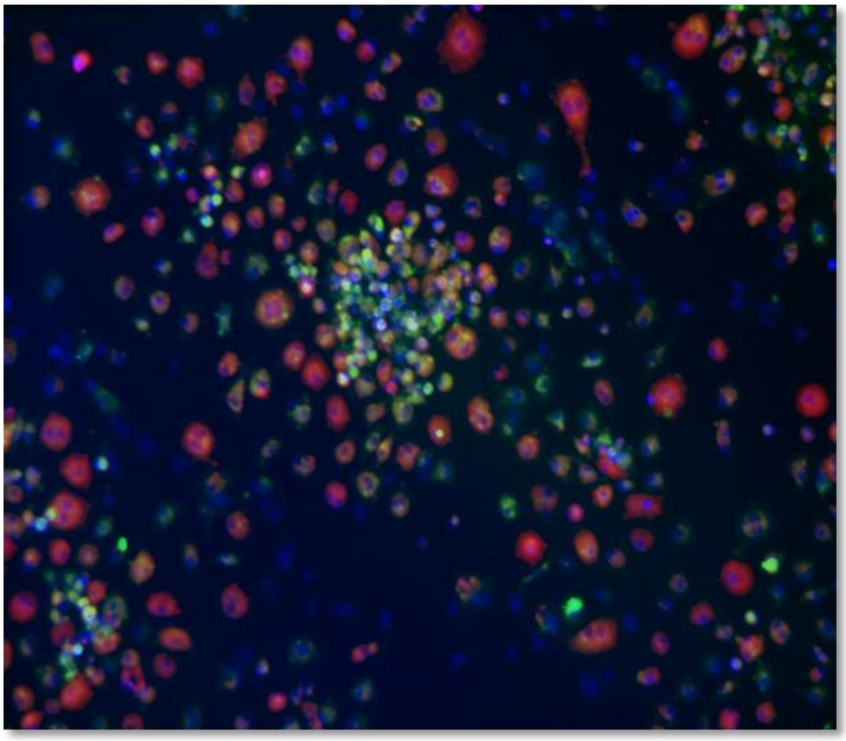


Fig 20b. eCFUs stained using DiI-acLDL(red) and lectin-FITC (green/yellow). Blue fluorescence denotes nuclear stain. (x10)

Table 8. The effect of changing the initial plating PBMNCs concentration on eCFU growth

Plating concentration (cells/ml)	**eCFUs mean(SD) n=4**	**Intra-Assay Variability**
5×10^6	30 (±3)	10%
2.5×10^6	18 (±2)	11%
1.25×10^6	0	-
6×10^5	0	-

Varying the concentration of FCS in EBM-2 from 5% to 20% did not significantly affect the CFU number (Spearman rank correlation R=0.31, P=0.68) (Table 9). The intra-assay variability was approximately 10%.

Table 9. The effect of changing FCS concentration on the growth of eCFUs.

Concentration of FCS in EMB-2	**eCFUs mean(SD) (n=4)**	**Intra-Assay Variability**
5% FCS	23.8 (±1.7)	7%
10% FCS	24.3 (±2.9)	12%
15% FCS	23.3 (±2.2)	9%
20% FCS	24 (±2.6)	11%

The inter-observer error was determined by growing eCFUs from five healthy volunteers. Two different observers were then asked to independently count the number of colonies in the same set of wells. The inter-observer error was <5% (Pearson correlation R=0.95, P=0.01, Fig 21)

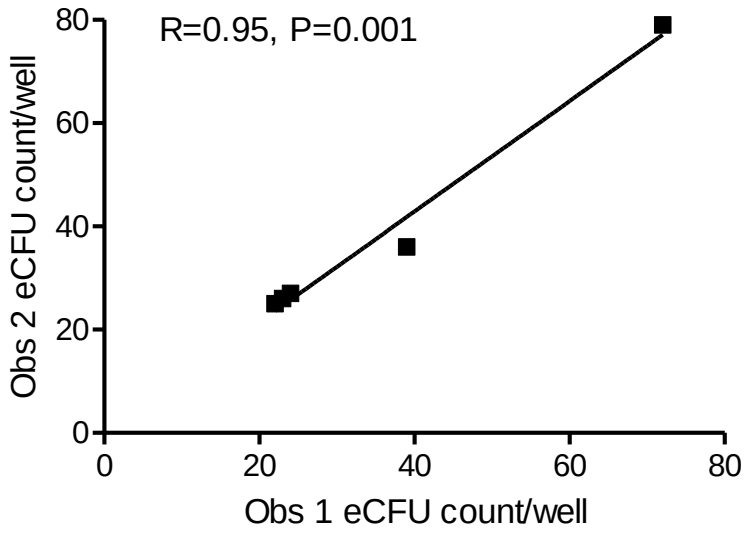


Fig 21. The inter-observer error of the eCFU assay (mean of n=3, Pearson correlation).

2.2.3.2 Culture of OECs/ late HPCs

From the published literature we were aware of the sequential changes that might be expected from the culture of eCFUs and subsequently OECs. In our culture, as presented in section 2.2.3.1, the appearance of early CFUs occurred between day 3 and 6 (Fig 22 A-C). After 10 days there was no obvious cobblestone appearance developing and a gradual loss of cells (Fig 22 D-F) which was contrary to published accounts[104]. We therefore investigated the changes in some of the assay conditions in order to grow OECs. These changes included (as per section 2.2.2.5):-

(i) changing the initial plating concentration of PBMNCs;

(ii) varying the concentration of FCS in the culture medium;

(iii) use of different batches of FCS;

(iv) pre-plating the PBMNC and culturing the non-adherent fraction at 48 hrs in 12-well plates (section 2.2.2.2 and 2.2.2.5).

OECs formed in less than 10% of MNC cultures from the same healthy volunteer (n=4 for each assay condition in 2.2.2.5) but was not reproducible for any given set of assay conditions. To date we have been unable to culture OECs from the peripheral blood in a reproducible manner.

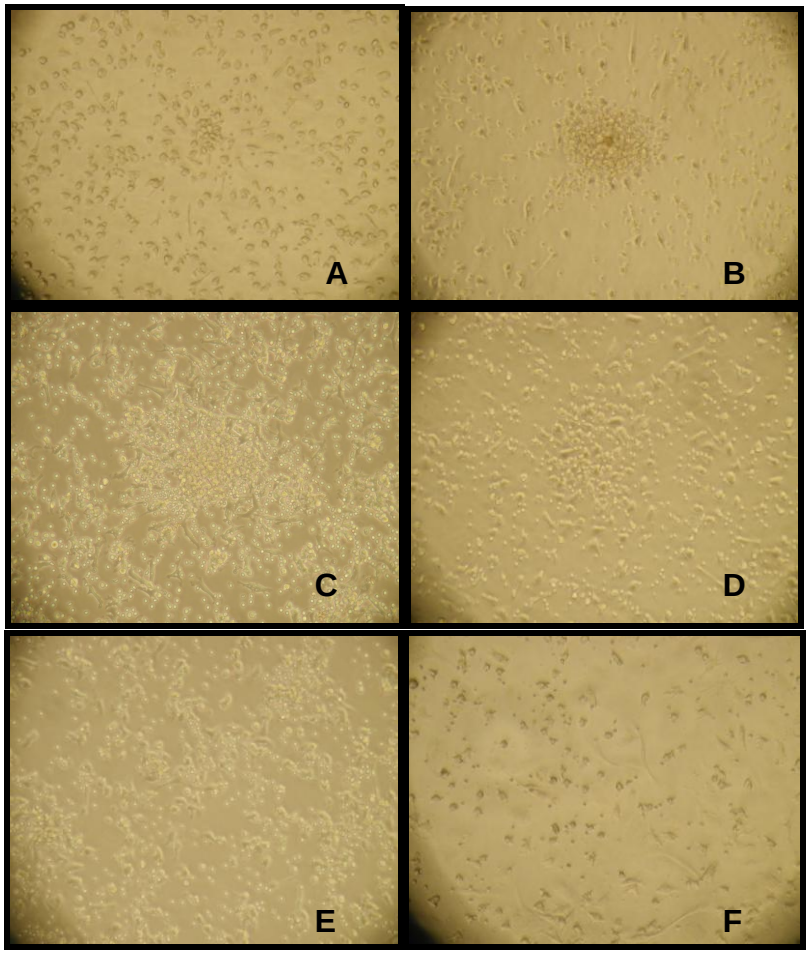


Fig 22. CFU formation over a 28 day period following seeding with isolated PBMCs (A) 2 days. (B) 4 days. (C) Six days - showing a typical early HPC-CFU. (D)10 days after plating. (E -F) 14 and 28 days - showing a gradual loss of cells and no cobblestone appearance (OECs).

2.2.4 Discussion

Numerous assays have been developed to culture mononuclear cells in order to isolate putative progenitor cells (PCs) that differentiate into ECs in-vitro and assay for colony forming capacity[92, 101, 111, 118, 121]. Two major cell types have been been shown to emerge from these cultures: (i) eCFUs, which display a mixed endothelial-haematopoietic/monocytic phenotype[109, 121, 128] and (ii) OECs (late CFU) cells with a high proliferative potential that display typical endothelial characteristics[110, 121, 122]. We have successfully managed to reproduce and optimise the eCFU assay by growing colonies of spindle shaped cells from MNCs. Furthermore we have shown that these cells express CD31, VEGFR2, DiI and Lectin after 5 days in culture, which are not expressed at day 1. These results appear to confirm evidence in the literature that a population of cells present in the initial plated fraction progress to an endothelial-like phenotype[101, 111, 132]. It also confirms that circulating endothelial cells (CEC) were not the source of our day 5 endothelial like cells.

OEC growth was not reproducible and was achieved in less than 10% of attempted cultures. Many groups have published on successful OEC growth from both peripheral blood and umbilical cord blood[110, 121, 122, 128]. Our attempts proved highly variable irrespective of the assay conditions employed.

2.2.5 Conclusions

- Adherent PBMCs did not stain with the EC markers (Dil/Lectin or CD31/VEGFR2) at 1 day. This suggests that HPCs and not circulating endothelial cells (CECs) were the origin of the colonies that formed by 5 days.
- eCFU growth did not occur below an initial plating concentration of 2.5×10^6 cells/ml. This concentration was therefore used in all subsequent assays.
- eCFU growth was highly dependent on the source of FCS and fibronectin
- eCFU growth did not depend on the percentage of FCS present in EBM-2 and therefore we have chosen to use 5%
- The intra-assay variability of the CFU assay was~10%
- Late HPC/OEC growth was highly variable.

2.3 HPC Function

2.3.1 General methods

2.3.1.1 HPC Migration Assay

The migratory capacity of HPCs toward a VEGF concentration gradient was examined using a modified Boyden chamber system[254].

i. Filters (1μm, BD Falcon HTS Fluoroblok) were coated in a solution of fibronectin (10μg/ml in M199 culture medium – Sigma, UK) at least 24 hrs before the assay. Before use, the filters were washed with DMEM and placed into 24-well plates (BD Falcon).

2 Isolated PBMCs were grown as per the eCFU assay procedure (2.2.2.2)

3 After 5 days in culture, the culture media was gently aspirated and the cells were washed twice with pre-warmed sterile PBS.

4 Cells were incubated with 3mls of DMEM supplemented with 2% charcoal treated FCS containing 5μg/ml of Calcein AM (Molecular Probes, UK) for 2hrs at 37^{o}C.

5 Cell detachment from the plate was carried out by first washing the cells twice with pre-warmed sterile PBS followed by addition of 1ml of Accutase® solution (Invitrogen) and incubation for 5 mins at 37^{o}C. The plate was gently tapped to encourage cells to detach.

6 The detached cells were treated with 2mls pre-warmed DMEM to neutralise the Accutase. The cell suspension was then transferred into sterile 15ml conical-bottom tubes (Falcon, BD).

7 The suspension was centrifuged at 400g for 5mins at 22 ^{o}C , and the pellet resuspended in pre-warmed DMEM and centrifuged once more at the same speed.

8 After a second wash the pellet was resuspended in pre-warmed DMEM containing 3.5mg/ml BSA and the cell numbers adjusted to 10^5 cells/ml.

9 300µl of the cell suspension was placed into the top chamber of each Fluoroblok insert.

10 Simultaneously, 1ml of the test solution (50ng/ml recombinant VEGF protein [R and D systems] in DMEM containing 3.5mg/ml BSA) or control solution (DMEM containing 3.5mg/ml BSA) was placed in the lower chamber.

11 The wells were incubated at 37^{o}C and fluorescence intensity measured every hour using a bottom reader fluorimeter (Perkin-Elmer Victor 3).

2.3.1.2 Senescence Assay

HPC senescence was measured by staining for senescence-associated β-galactosidase activity [255].

1. Isolated PBMCs were grown as per the CFU assay procedure in fibronectin-coated chamber slides (Nunc) (see 2.2.2.2). After 5 days in culture, the cells were washed twice with 1ml warmed sterile PBS.

2. 1ml of pre-warmed 300µM chloroquine (ImaGene Green Kit, Molecular Probes) in EBM-2 medium (Cambrex) was added to the cells in each well and incubated for 1hr at 37^{o}C.
3. Neat C12FDG (ImaGene Green Kit – Molecular Probes) substrate was added to the chamber slide directly to achieve a final concentration of 33µM, the solution was gently mixed, and incubated for 1hr at 37^{o}C.
4. The cells were washed twice with pre-warmed PBS before being fixed with 2% paraformaldehyde in PBS for ten minutes.
5. The slide was washed twice with PBS.
6. The chambers were removed and the slide mounted with Vectorshield containing DAPI.
7. The slides were viewed under a fluorescent microscope. HPC senescence was quantified as a percentage of the total number of cells staining positive for β galactosidase.

2.3.1.3 Optimisation experiments

The following experiments were carried out to optimise the migration assay (section 2.3.1.2).

- Use of microvascular endothelial cells (MVEC's) as positive controls.
- Use of 0.5mmol EDTA, 1%Trypsin EDTA, and Accutase to determine the optimal way of detaching the cells from culture.

- Comparison of different concentrations of VEGF to induce optimal migration (25, 50, 75ng/ml).

2.3.2 Results

2.3.2.1 Migration Assay

A typical curve produced from the migration of cultured HPCs (from a healthy volunteer) towards VEGF or a control solution is shown in Fig 23. It can be seen that there is migration of HPCs in the absence of a chemotactic agent such as VEGF (control curve). Addition of VEGF (50ng/ml) to the solution in the bottom chamber significantly increases the migration rate of HPCs ($P<0.001$, ANOVA). Migration appeared, however, to equalise by 24hrs. The effect of the chemotactic stimulus was therefore expressed as a percentage increase in the fluorescence in response to the VEGF solution compared to the control solution at 4hrs (peak migration rate). The intra-assay variability for this was 12% (n=6 HPC isolates, each in triplicate).

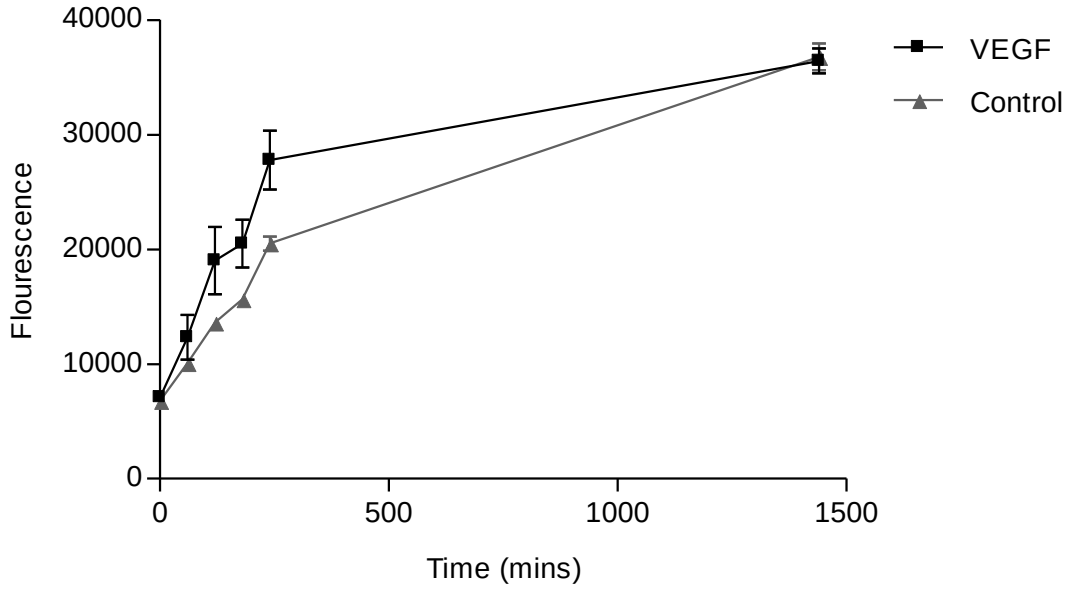


Fig 23. A typical migration curve for HPCs from a single patient showing positive (VEGF 50ng/ml) and control curves (n=3 at each point).

The effect of varying the concentration of VEGF on the migration of HPCs cultured from healthy volunteers is shown in Fig 24. The highest concentration of VEGF (75ng/ml) induced the greatest migratory response (VEGF:control fluorescent ration of 1.34, i.e an increase of 34% over control, $P<0.001$, ANOVA), with no response over control levels from the lowest concentration of VEGF, (25ng/ml, 1% migration, $P=0.43$, ANOVA). There was no difference in the migration of HPCs between 50ng/ml and 75ng/ml VEGF ($P=0.73$, ANOVA).

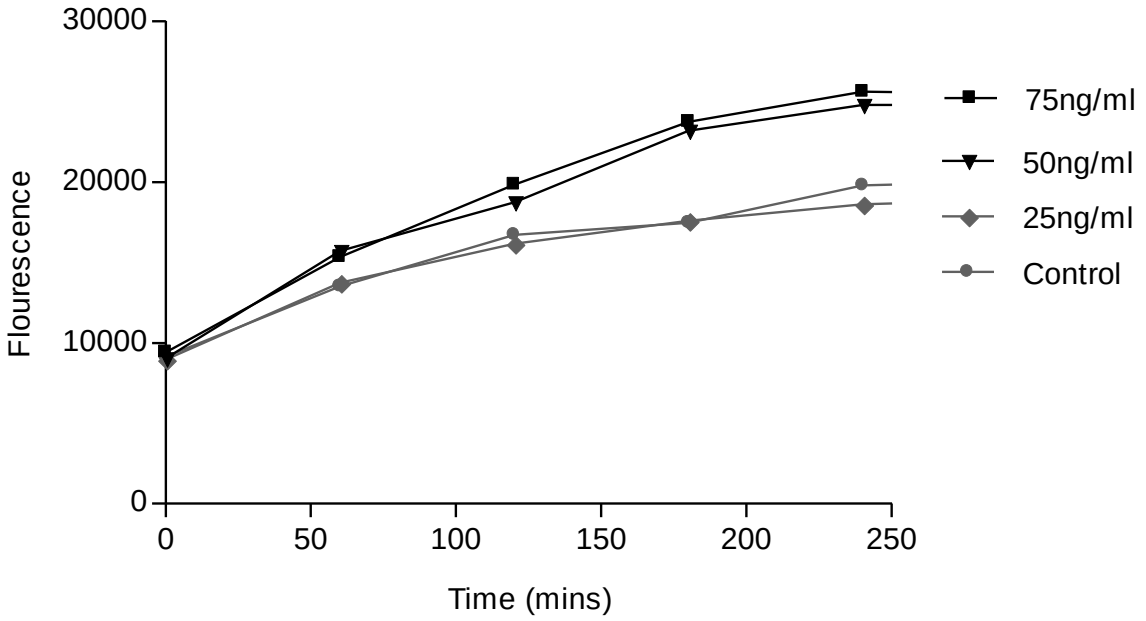


Fig 24 The effect of varying the concentration of VEGF on migration of HPC's (mean of triplicates at each concentration).

2.3.2.2 Senescence assay

Fig 25 shows a typical stain for SA-Bgal activity in cultured HPCs. Only cells brightly fluorescent green were counted as positive for B-galactosidase. The B-galactosidase positive (green) and total number of cells (staining with the nuclear stain DAPI) were counted in 10 random fields. The number of B-gal positive cells was then expressed as a percentage of the total number of cells. The intra-assay variability was 15% (6 different slides grown from the same healthy volunteer). The inter-observer error (correlation between two observers over 5 different slides of HPCs each grown from a different healthy volunteer) was 12% (Pearsons correlation R= 0.88, P=0.04).

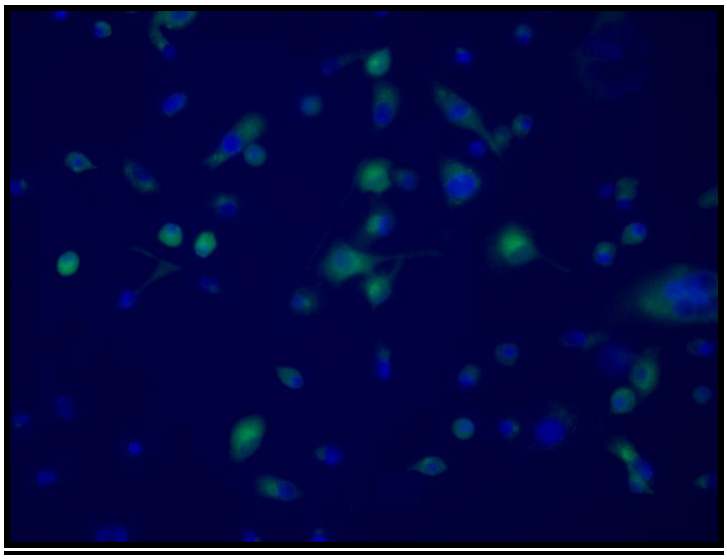


Fig 25. C12FDG stain showing senescent HPC's (green) after 5 days in culture.

2.3.3 Discussion

The isolated HPCs in this study were shown to migrate to a VEGF chemotactic stimulus. There was a threshold concentration at which migration was increased, which was 50ng/ml. Above this concentration the overall migration did not increase significantly with increased concentrations of VEGF. By 24hrs the migration of HPCs in both the VEGF and control wells were similar, suggesting that all cells had passed through the filter by this time. The baseline migration in the control wells clearly shows that these cells may migrate through the filter in the absence of a stimulus. HPCs are known to secrete a number of growth factors such as VEGF and SDF-1[256]. These factors may have stimulated their slow migration in an autocrine manner, which could be enhanced in the presence of the recombinant

VEGF, acting as a supraphysiological stimulus. Alternatively in the absence of a VEGF concentration gradient, HPCs may have migrated randomly across the fibronectin, (used to coat both sides of the 1µm filter) and with time, would have accumulated on the underside of the filter.

2.3.4 Conclusion

- HPCs migrate in response to VEGF, but there appeared to be no improvement in this response above 50ng/ml.
- The migration was greatest at 4hrs, and equilibrates with that of the controls by 24hrs.
- The intra-assay variability of the migration assay was 12%.
- The intra-assay variability of the senescence assay was 15%

2.4 Mesenchymal Stem Cells

2.4.1 Overview of investigations

2.4.1.1 MSC number

Whole blood was stained as per the direct immunofluorescence protocol (2.1.2.1) using antibodies against CD34 – PerCP and CD45 – FITC, and either CD73-PE, CD90-PE or CD105-PE, in order to detect cells that were $CD45^{-ve}/CD34^{-ve}/CD73^{+ve}/CD90^{+ve}/CD105^{+ve}$ i.e. identified as being of non-haematopoietic lineage, and analysed by flow cytometry. MSCs were defined as those cells that were $CD45^{-ve}/CD34^{-ve}$ and $CD73^{+ve}$ or $CD90^{+ve}$ or $CD105^{+ve}$ [187] .

2.4.1.2 MSC activity

Isolated mononuclear cells were plated onto plastic dishes in DMEM culture medium containing 10% FBS. Adherent cells were incubated and passaged for up to four weeks[190]. Following culture cells were analysed by flow cytometry to see if they expressed the MSC phenotype. Senescence was to be measured as described above and migratory activity assessed using a PlGF concentration gradient [257] in a modified Boyden Chamber.

2.4.2 General methods

2.4.2.1 Mesenchymal Progenitor Cell Culture

1. PBMNCs were isolated by Ficoll gradients as per 2.2.2.1.

2. The pellet in step 7 was resuspended in low glucose DMEM (Cambrex) containing 10% foetal calf serum, 1% penicillin/Streptomycin (MSC medium), to a volume one quarter the original volume of blood.
3. The number of cells was counted using a haematocytometer, and the concentration adjusted to 5 x 10^6 cells per ml.
4. The cell suspension was incubated in 6 well plates (4mls/2x10^7 cells per well) in a 5% CO_2 incubator at 37°C.
5. The non-adherent fraction was removed at 48 hrs by gently aspirating the culture medium and replacing it with 2mls pre-warmed MSC medium.
6. The medium was changed every 4 days thereafter.
7. When the cells were 80% confluent they were passaged. Cell detachment from the plate was carried out by first washing the cells twice with pre-warmed sterile PBS followed by addition of 1ml of Accutase® solution (Invitrogen) and incubation for 5mins at 37^{o}C. The plate was gently tapped to encourage cells to detach.
8. The detached cells were treated with 2mls pre-warmed DMEM to neutralise the Accutase.
9. The cell suspension was then transferred into sterile 15ml conical-bottom tubes (Falcon, BD) and centrifuged at 400g for 5 mins at 22ºC. The pellet was resuspended in DMEM.
10. After a second wash the pellet was resuspended in MSC medium and adjusted to a concentration of 4 - 6 x 10^4cells/ml.

11. 4mls of the suspension was placed into each 25 ml flask, and incubated in a 5% CO_2 incubator at 37°C.
12. The cultured was continued as above.

2.4.2.2 MACS magnetic labelling

1. PBMNCs were isolated by Ficoll gradient as per 2.2.2.1.
2. The PBMNC pellet (step 7 2.2.2.1) was re-suspended in autoMACS running buffer (Miltenyi) to a volume one quarter the original volume of blood.
3. The cells were counted using a haematocytometer and the concentration adjusted to 5 x 10^6 cells per ml.
4. The cells were passed through a 30µm nylon filter to remove clumps.
5. The primary antibody was added at the recommended dilution (mouse anti-human CD45 primary, Dako, 1:400) to the cell suspension, which was mixed well and incubated at 4ºC for 30 mins.
6. The cells were washed by adding 10-20 times the labelling volume of the MACS buffer, centrifuged at 400g for 5 mins at 22ºC, after which the supernatant was removed.
7. The wash step was repeated three times.
8. The pellet was re-suspended in 80µl of MACS buffer per 10^7 total cells.
9. 20µl of MACS indirect IgG Microbeads (goat anti-mouse microbeads - Miltenyi) was added to 10^7 cells. The suspension was mixed well and incubated for 15 mins at 6 -12ºC.

10. Cells were washed as per step 6, three times and finally resuspended in 500µl MACS buffer per 10^8 total cells.

2.4.2.3 MACS magnetic separation

1. An appropriate column type was chosen (MS for up to 10^7 total cells or LS for up to 10^8 total cells) and placed in the magnetic field of a suitable MACS separator.
2. The column was prepared by washing with an appropriate amount of MACS buffer (MS – 500µl, LS 3mls).
3. The cell suspension was pipetted into the top of the column. The cells that have not been labelled pass through. Once the cell suspension has passed through, the column was rinsed with appropriate amount of MACS buffer (MS 3x 500µl, LS 3x3mls).
4. The filtered cell suspension was then centrifuged at 400g for 5 mins at 22ºC, after which the supernatant was removed and the cells resuspended in MSC medium.

2.4.2.4 Optimisation Experiments for Cell Culture

The following experiments were carried out to determine the optimal cell culture conditions.

- The initial plating concentration of PBMNCs in step 3(2.4.2.1) was varied (5 x 10^6, 2.5 x 10^6, 1.25 x 10^6, 6 x 10^5 cells/ml).

- The concentration of FCS in the MSC medium was varied (2.4.2.1 step 2 - 20%, 10%, 5%)
- Batch testing FCS (from Sigma, Gibco, Hyclone)
- Complement depletion of FCS (heating for 30 mins at 60°C)
- CD45 cell depletion using MACS microbead cell separation (CD45 mouse anti-human primary, goat anti-mouse microbeads secondary) followed by MSC culture.

2.4.3 Results

2.4.3.1 Measuring MSCs by flow cytometry

Whole blood from healthy volunteers was stained as per the direct immunofluorescence protocol (2.1.2.1) in order to detect MSCs. Flow cytometric analysis revealed that after the $CD34^{-ve}/CD45^{-ve}$ cell fraction was selected (Fig 26a) we were unable to detect any cells expressing CD90 (Fig 26b). We also failed to detect $CD34^{-ve}/CD45^{-ve}/ CD73^{+ve}$ cells (Fig 26c) or $CD34^{-ve}/CD45^{-ve}/CD105^{+ve}$ cells (Fig 26d). Varying the antibody concentration, incubation temperature or incubation time had no effect on the detection of MSCs in the peripheral circulation. We were therefore unable to measure MSCs by flow cytometry.

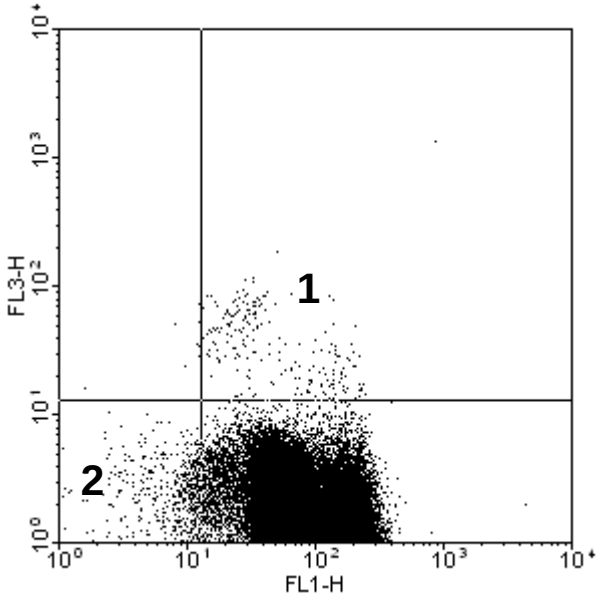


Fig 26a. Dot plot of CD45-FITC (FL1) versus CD34-PerCP (FL3)

Area **1** contains cells that are double positive for CD45 and CD34

Area **2** contains cells that are double negative for CD45 and CD34

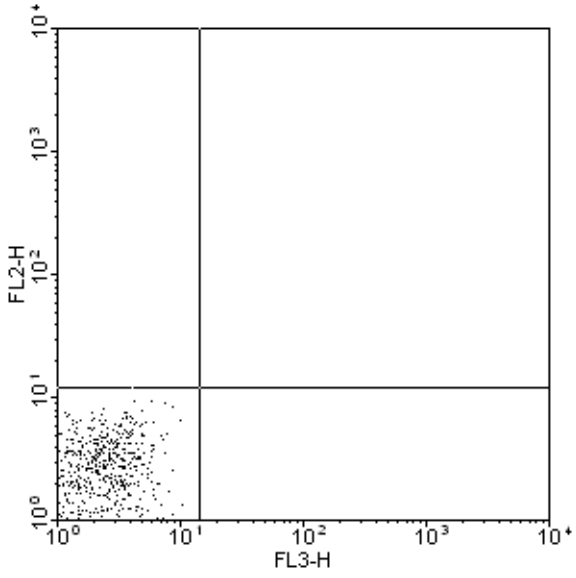


Fig 26b. Dot plot of CD34 - PerCP (FL3) versus CD90 - PE (FL2)

Only cells that were CD34 and CD45 negative (area 2 in Fig 26a) are shown.

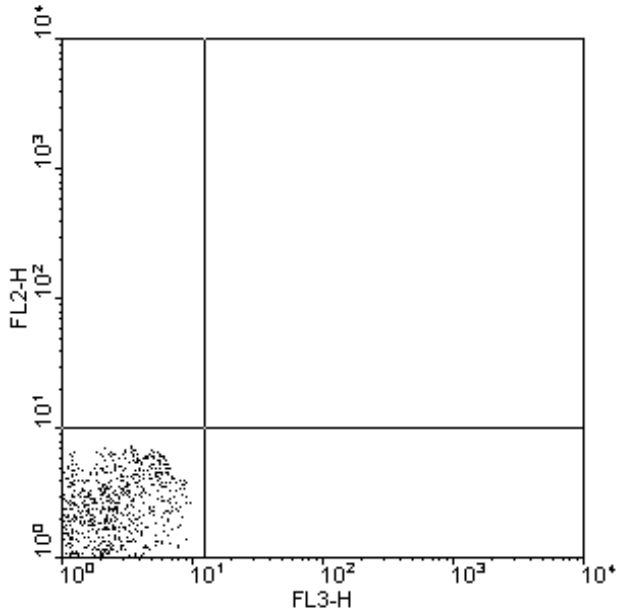


Fig 26c. Dot plot of CD34 - PerCP (FL3) versus CD73 - PE (FL2)

Only cells that were CD34 and CD45 negative (area 2 in Fig 26a) are shown.

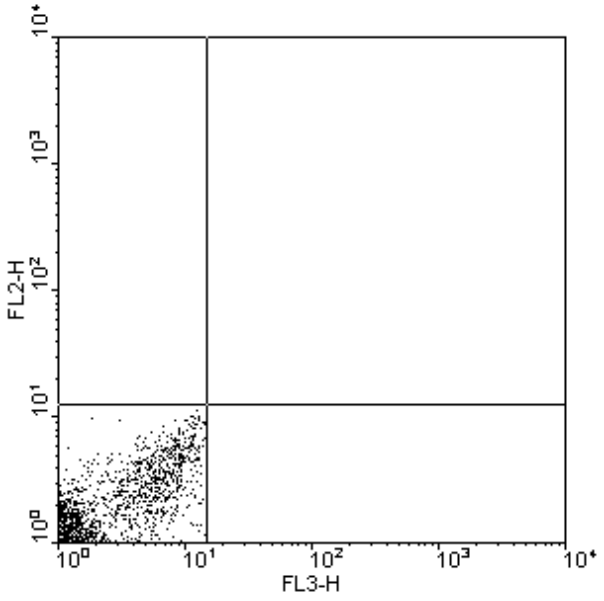


Fig 26d. Dot plot of CD34 - PerCP (FL3) versus CD105 - PE (FL2)

Only cells that were CD34 and CD45 negative (area 2 in Fig 26a) are shown.

2.4.3.2 Mesenchymal Progenitor Cell Culture

MSC isolation was attempted by culturing peripheral blood MNCs from healthy volunteers (2.4.2.1). The cultured cells (Fig 27) did not demonstrate the rapid proliferative characteristics as described in the literature, even after cell passage. FACS analysis of 4 week old cells (Fig 28) that had been passaged once showed that all cells were CD45 positive and therefore not MSCs. In order to rule out experimental error the following experimental conditions were altered: initial plating concentration of PBMNCs; the concentration of FCS in the culture medium, and source of FCS; complement depletion of serum; and depletion of leukocytes using CD45-linked MACS microbead cell separation. None of these changes resulted in the growth of MSCs.

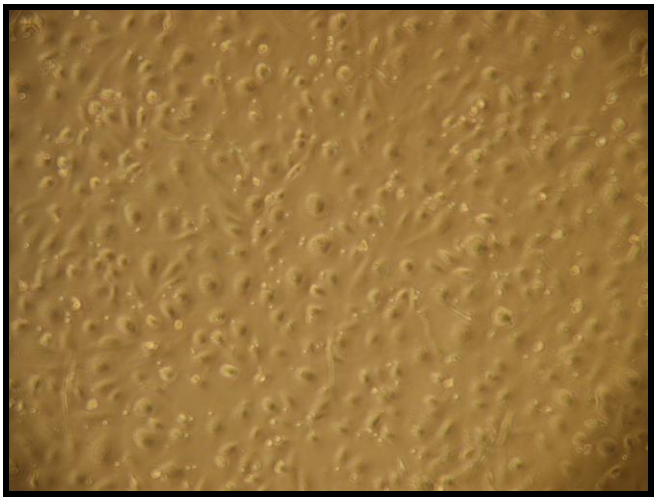


Fig 27. Light microscope picture of cells (x20) from MSC culture (day 14). Cells show a cobblestone appearance and did not proliferate after passage.

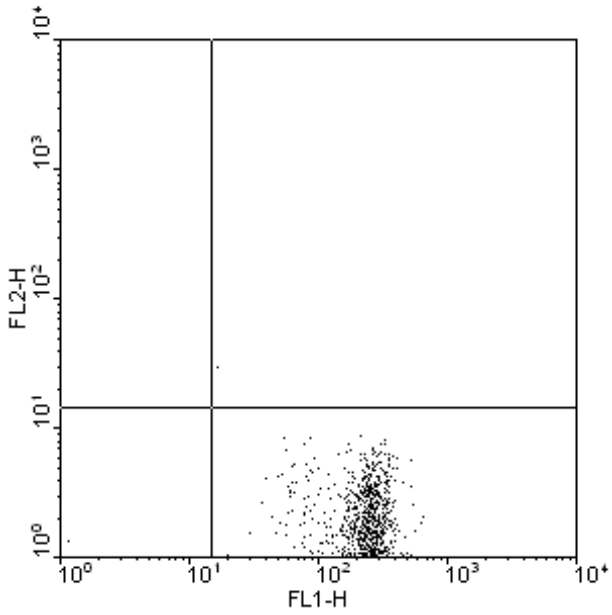


Fig 28. Dot plot of CD45-FITC(FL1) versus CD90-PE(FL2). All cells were CD45 +ve and none were CD90 +ve.

2.4.4 Discussion

The culture of mononuclear cells on plastic dishes in DMEM containing 10% FBS and subsequent passage[190] has been shown to result in a population of fibroblast-like cells, which have been characterized as MSCs based on their ability to differentiate into multiple mesenchymal lineages (e.g. osteogenic, chondrogenic, myogenic, etc.) [190]. We have been unable to measure MSCs in the circulation through FACS analysis or using the same cell culture technique. There are several possible reasons why this might have occurred. The methods used for their detection were those previously used to identify MSCs in the bone marrow[190, 258]. Groups that have identified them in the circulation have done so after clinical

events that stimulate mobilisation from the bone marrow such as myocardial infarction[259] or fractures[260]. The latter study also failed to identify them in the control groups of pre- and post-menopausal women, and elderly patients with osteoarthritis. Our group of patients and healthy volunteers may simply have no circulating MSCs or have so few that they lay below the detection threshold of our measurement systems. Alternatively there may have been a methodological problem that prevented us from detecting these cells. Positive controls are sometimes used in these instances. We had access to patients with long bone fractures that according to the literature would have been more likely to have circulating MSCs [260], but even in this cohort, less than half of the patients have raised circulating MSC numbers. This inconsistency would have introduced variability into our analyses making interpretation of results difficult if these patients were used as positive controls.

2.4.5 Conclusion

- We were unable to measure MSCs in the peripheral blood by flow cytometry or cell culture.
- This may be as a result of their low numbers in the circulation which lies below the detection threshold for our assays
- This may also be a result of methodological problems which we were unable to resolve because of the lack of a consistent positive control

2.5 Cytokine measurement

The plasma concentrations of the cytokines VEGF, GM-CSF, SDF-1, and PLGF were analysed by multiplex ELISA (Searchlight, Pierce Biotechnology, UK). SearchLight Protein Array Technology is a multiplexing sandwich-ELISA system based on chemiluminescent detection of analytes whose respective capture-antibodies are spotted in arrays within each well of a 96-well microplate. The protein binding to the capture antibody in an array can be detected directly. As many as 16 analytes (4 x 4 array in each well) can be measured per well, meaning that 16 cytokines or other biomarkers can be assayed simultaneously with each 50 µl sample.

Compared with traditional ELISA, multiplex arrays have a number of advantages [261] including:-

a) high output multiplex analysis

b) less sample volume needed

c) efficiency in terms of time and cost

d) ability to evaluate the levels of one given inflammatory molecule in the context of multiple others

e) ability to reliably detect different proteins across a broad dynamic range of concentrations.

We used the custom sample testing service which allowed us to select individual cytokines. 4mls of sodium citrate anticoagulated blood was centrifuged at 1500g at 4ºC for 15 mins (brake off). The plasma fraction was siphoned off and aliquots of

0.25mls were placed in eppendorf tubes. Samples were snap frozen in liquid nitrogen and stored at -80 ºC until analysis.

2.6 Quantification of risk of further stroke

The Oxford Risk Tables (www.stroke.ox.ac.uk) were used to predict the 1yr and 5yr absolute risk of ipsilateral ischaemic stroke (as a percentage) in patients with a recently symptomatic carotid stenosis. The program is based on the results of Cox regression model of patients who had been randomised to medical treatment in the European Carotid Surgery Trial (ECST)[13, 262]. This involves five variables that were both significant predictors of risk in the ECST model [262] and yielded clinically important subgroup treatment effect in pooled analysis[16]. The model from which the tables were derived have been validated against the NASCET trial[16]. The five variables are: sex, age, time since last symptomatic event, type of presenting event and carotid plaque surface morphology. The risk prediction produced by the model is not a prediction of likely benefit from endarterectomy. The procedural risk and the 1-2% annual risk of stroke after successful treatment [12] must both be considered.

2.6.1 Oxford risk tables definitions

Degree of stenosis This refers to the maximum degree of linear stenosis at or around the symptomatic carotid bifurcation by method of measurement used in the NASCET trial and the Carotid Endarterectomy Trialists' Collaboration [12].

Time since last event: refers to the most recent ipsilateral vascular symptomatic event. Accepted values for this variable are limited to between 7 days and 180 days.

Primary symptomatic event. This refers to the most "severe" ipsilateral vascular event during the previous 6 months (major stroke > minor stroke > multiple cerebral TIAs > single cerebral TIA > monocular TIA or retinal artery occlusion).

Major stroke - defined as a non-disabling stroke with residual neurological symptoms after 7 days.

Minor stroke - defined as a stroke with symptoms lasting between 24 hours and 7 days.

TIA - defined as an event with symptoms lasting up to 24 hours.

Plaque surface morphology- irregular or ulcerated plaque surface. This is based on the surface morphology of the symptomatic carotid plaque as visualised on conventional arterial angiography, which was the imaging investigation of choice in the ECST. A patient with strong evidence of lipid rich or unstable/ruptured/ulcerated plaque on non-invasive imaging can also be entered

as having irregular or ulcerated plaque. Imaging is used as a substitute to histology in the program based on evidence that angiographically irregular and ulcerated plaque has been shown to be highly correlated with lipid-rich unstable or ruptured plaques on histology[263] The program assumes that it will be used by clinicians/surgeons before CEA and therefore the actual morphology of the plaque will not be known. Since all patients in our study underwent CEA, we were able to enter data on the exact morphology of the plaque.

2.7 Study Design

St Thomas' Hospital forms part of the Guy's and St Thomas' NHS Foundation Trust and is a tertiary referral centre for the treatment of a variety of vascular diseases. In excess of 50 carotid endarterectomies (CEA) are carried out each year. The aim of this study was to recruit sufficient patients over a 2-year period to provide a sample of at least 10-15 restenotic patients. We planned to collect a sample of 30mls of peripheral blood on the morning of the procedure, and 1 day and 6 weeks post procedure, and measure HPC numbers and activity. Blood was collected in EDTA anticoagulated tubes and processed for flow cytometry, functional assays or plasma extraction within 1 hour of collection. Plasma samples were snap frozen and stored at -80ºC.

2.7.1 Patient group

All patients undergoing primary CEA at St Thomas' were eligible for inclusion. Subsequent exclusion criteria are outlined below. The study was carried out under existing ethics approval 'Tissue and blood samples for the study of aortic and peripheral vascular disease - pathogenesis and circulating markers (occlusive disease) – EC 03/098 St Thomas' Hospital Ethics Committee'. (Appendix A). Patients were identified from the theatre lists and approached 24 hrs prior to the proposed collection of the first blood sample (i.e the day before surgery). The patient was provided with an information sheet and informed consent was obtained.

Symptomatic patients were defined as those that had experienced a sudden onset of loss of global or focal cerebral function, which was either permanent (stroke) or lasted less than 24 hours (TIA - including amaurosis fugax). Asymptomatic patients were defined as those who had never experienced symptoms. All patients had an internal carotid artery stenosis >70% demonstrated via duplex ultrasonography using conventional criteria[264].

2.7.2 Duplex assessment of restenosis

All patients who have CEA at St Thomas' Hospital currently have surveillance duplex scanning at 3, 6 and 12 months as part of their routine clinical follow-up. Internal carotid stenosis was graded from the absolute peak systolic and diastolic velocities in the ICA and the ratio of peak systolic velocities in the ICA and common carotid artery (CCA)[264]. These criteria have been validated against angiographicaly measured stenosis using the NASCET technique[265] and recently formed part of the recommendations for the reporting of ultrasound investigations of the extra cranial arteries produced by a Joint Working Group formed between the Vascular Society of Great Britain and Ireland, and the Society for Vascular Technology of Great Britain and Ireland[266]. PSV ratios (ICA/CCA) of 2.0, 2.5 and 3.4 were used to identify stenoses of 50, 60 and 70 per cent respectively. PSV ratios of >4 and >5 identified stenoses of >80 and >90 per cent respectively [264, 266]. The data on the degree of restenosis at the different time intervals was not collected until the end of the study period.

2.7.3 Exclusion criteria

Acute/ chronic inflammation as evidenced by a raised white cell count or C-reactive protein, subsequent episode of acute coronary syndrome or myocardial infarction, subsequent episode of critical lower limb ischaemia as defined by the onset of rest pain, non healing ulcers > 4 weeks, and any other surgery in the 6 weeks following CEA.

2.7.4 Data collection

All data was collected prospectively and included (i) clinical details such age, sex, co-morbidities, medication, presenting complaint that led to endarterectomy and degree of ICA stenosis; (ii) haematological variables such as differential leukocyte count, renal function and serum cholesterol and (iii) procedural details such as the use of patch repair, shunt and general or local anaesthesia. (Appendix B).

2.7.5 Data analysis and power calculation

The numbers of progenitor cells, their activity and the change in these end points over time were correlated with the presence of restenosis. All statistical analysis was carried out using SPSS 14.0 and all data tested for normality. Parametrically distributed data are given as mean ± standard deviation (SD) for population data or standard error of the mean (SEM) for sample data and analysed using either t-test or paired t-test as appropriate. Non-parametrically distributed data are given a median with ranges and analysed by Mann Whitney U test, or Wilcoxon Signed Rank test (if paired). Correlations were carried out by Spearman rank or Pearson rank as appropriate. Analysis by ANOVA was used when comparing temporal changes. Multivariate analysis was used to analyse associations between clinical (such as diabetes, smoking and hypertension) and haematology variables described above, with restenosis rates.

The number of patients (n=15) with restenosis post CEA is sufficient to give a power of 80-90% to detect a difference between groups with a statistical significance of $p<0.05$. This power calculation is based on an approximation of variation (~up to 30%) in circulating HPC numbers taken from the literature [267]. A more than 30% difference in HPC numbers between restenosis and non-restenosis groups are considered important differences. The incidence of restenosis (>50%) is 10-20% in the first year following CEA[29-31]. Thus a total number of between 60-100 patients was required to detect 15 patients who develop restenosis within the study period.

3. MEASURING HPC NUMBER AND FUNCTION IN PATIENTS UNDERGOING CAROTID ENDARTERECTOMY

A total of 62 patients were recruited to the study. Patient recruitment began when the methodology for the FACS detection of $CD133^{+ve}/CD34^{+ve}$ had been optimised. The HPC-CFU, migration and B-galactosidase assays were optimised later and this led to an initial cohort of patients (n=15) in which there were incomplete assay data sets. Figures 29 and 30 summarise the investigations performed on all 62 patients.

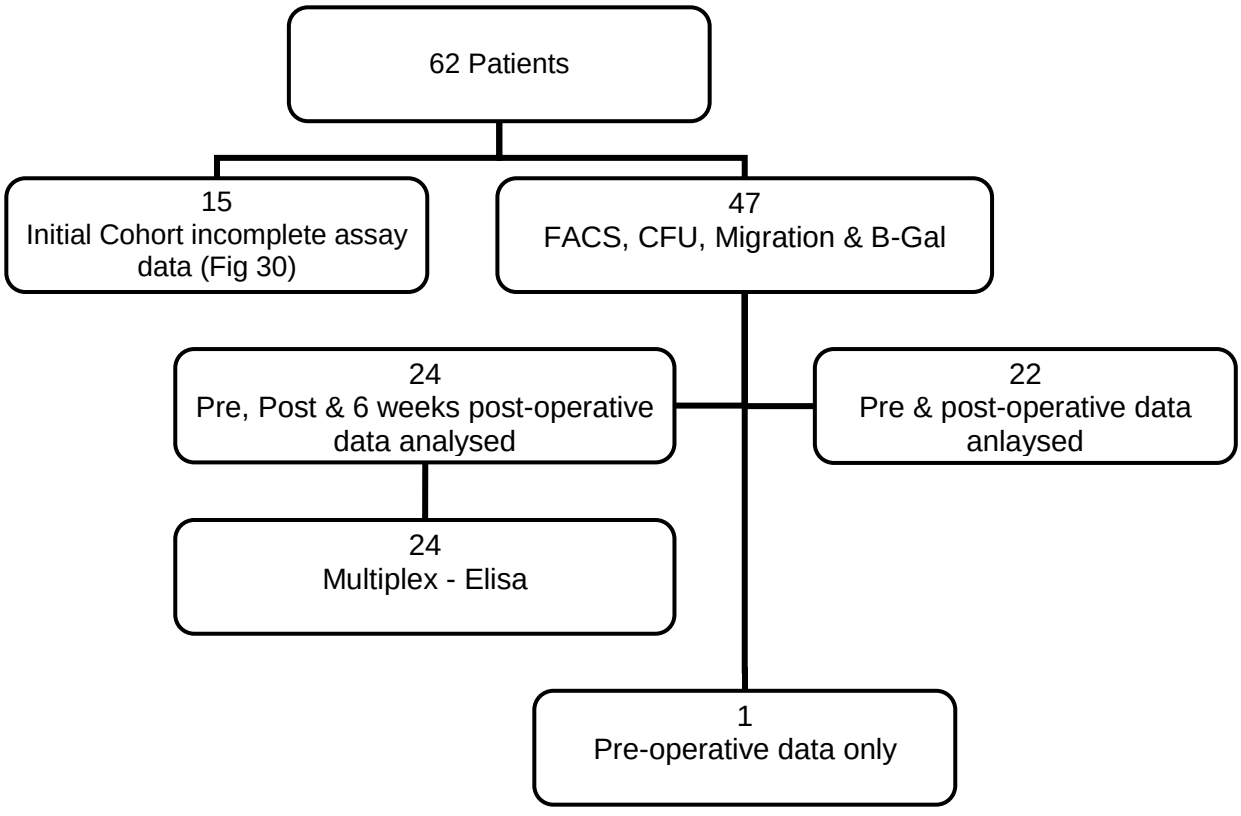


Fig 29. Flow Diagram of the investigations performed on the 62 patients recruited to the study.

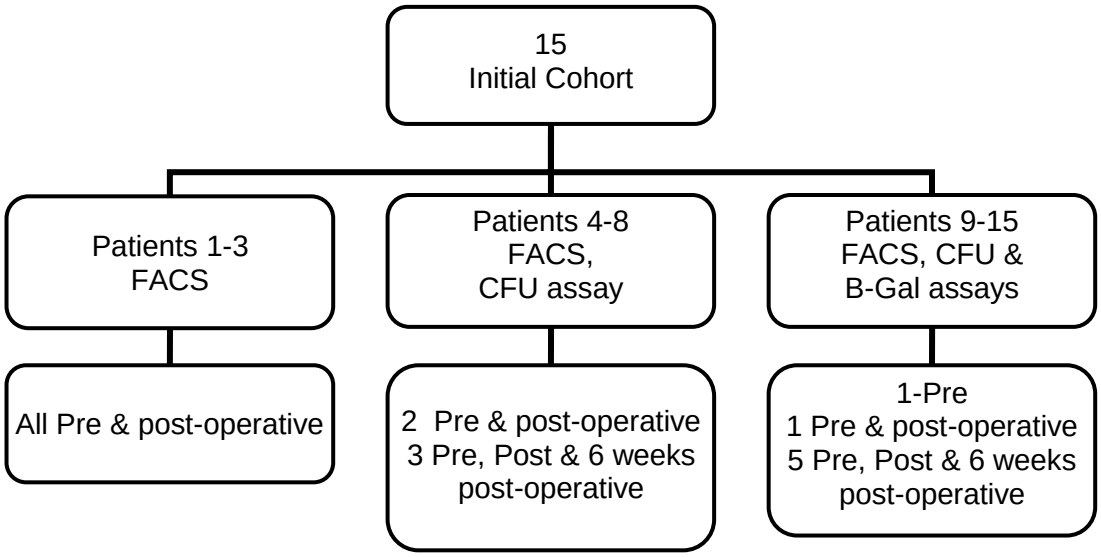


Fig 30. Flow diagram of the investigations performed on the initial cohort of 15 patients recruited to the study

The first 15 patients all had FACS analysis of circulating CD133+/CD34+ cell number and also had other investigations as outlined in Figure 30. The investigations were carried out at the three different time points (pre-operatively [morning of operation], 24 hrs post-operative and 6 weeks post-operative) in 8 patients, and in a further 6 only at the pre and post-operative time points. The latter 6 patients either refused to have the 6 week blood sample taken (4) or did not attend the follow up appointment at all (2). A further patient had blood taken but the cultures became infected and therefore further analysis was not possible.

The following 47 patients recruited to the study had the full set of investigations i.e. FACS analysis, eCFU, migration and ß-galactosidase assays, but varied by the

number of time points that this set of investigations were done (Fig 29). 24 patients had the full set of investigations performed at the three different time points i.e pre and post-operatively and 6 weeks post-operatively. A further 22 patients had the full set of investigations at the pre- and post-operative time points, but not at the 6 week follow up. These 22 either refused to have further blood taken at the 6week follow up (12) or did not attend the 6 week follow-up (10). A single patient having had the full set of investigation on the pre-operative sample refused to have any further blood taken. The numbers included in the following analysis will therefore vary and reference should be made to Figures 29 and 30 for clarification.

3.1 Patient demographics and procedural details

The 62 patients recruited to the study had a mean age of 71yrs (±8.5SD) and consisted of 44 males and 18 females. 20 patients had suffered from strokes and 20 from transient ischaemic attacks (including amaurosis fugax) within the 6months prior to CEA. 22 patients were classified as asymptomatic in the last 6 months. Of these patients 18 had never experienced symptoms. The mean stenosis of the internal carotid artery was 79%(±11) on the ipsilateral side and 56%(±25) on the contralateral side to endarterectomy. The mean time from symptoms to endarterectomy was 7.4 weeks (±11.5).

The atherosclerotic risk factors in the study group are given in Table 10. Hypercholesterolaemia (92%), smoking (73%) and hypertension (76%) were the most predominant atherosclerotic risk factors present. All patients were taking anti-

platelet agents aspirin (46) or clopidogrel (16). The cholesterol lowering statins were taken by 57 of the 62 patients, with the remaining 5 having stopped them in the past because of side effects. Patients were also on a number of anti-hypertensive medications including β-blockers (27), diuretics (21), ACE inhibitors (28), angiotensin II receptor antagonists (7) and Ca channel antagonists (24). All patients with hypertension were on 1 or more antihypertensive medications.

Table 10. Atherosclerotic risk factors in patient group

Risk Factors	% of patients
Hypertension	76
Diabetes	24
Ischaemic Heart Disease	43
Smoker	73
High Cholesterol	92
Peripheral Vascular Disease	19
Renal Failure	7

Pre-operative differential leukocyte counts are given in Table 11. Comparison of leukocyte counts pre and post-CEA showed that there was a rise in total leukocyte numbers ($x10^9$cells/L, mean±SD), 8.2(±2.2) n=62 to 10.4(±3.4) n=53, P<0.001). Cell-type subanalysis revealed a significant increase in neutrophil numbers

(5.4(±2.2) to 8.0(±3.5), P<0.001); a reduction in lymphocyte numbers (1.9(±0.72) to 1.5(±0.48), P<0.001); and a small, but non-significant increase in the monocyte count (0.67 (±0.18) to 0.72(±0.32), P=0.28, all paired t-test).

Table 11. Pre and 24 hr post-operative blood results (*Paired t-test).

	Pre-op (mean SD)	Post-op (mean SD)	P value
Total leukocyte ($x10^9$ cells/L)	8.2(2.2)	10.4(3.4)	<0.001
Neutrophils ($x10^9$ cells/L)	5.4(2.2)	8.0(3.5)	<0.001
Lymphocytes ($x10^9$ cells/L)	1.9(0.7)	1.5(0.5)	<0.001
Monocytes ($x10^9$ cells/L)	0.67(0.2)	0.72(0.3)	NS

The majority of CEAs (84%) were carried out under general anaesthesia. There was a slight predominance of left sided CEA (61%). Patch angioplasty following endarterectomy was performed in 63% of patients and only 37% of patients needed an intra-operative carotid shunt (Table 12). There was one stroke and one TIA intra-operatively, and one patient had to return to theatre because of post-operative bleeding. There were no mortalities or other complications within the 12 month follow–up period.

Table 12 Procedural characteristics

Procedure	% of patients
Left side	61
Patch	63
General Anaesthetic	84
Shunt	37

3.2 Association between HPC number and function and clinical parameters

There was an 11.3(±4.7)% 1 year risk and a 27.8(±11)% 5 year risk of a further ipsilateral ischaemic stroke for symptomatic patients (n=40) as assessed using the Oxford tables. There were no correlations between the 1yr and 5yr risk of stroke and pre-operative circulating $CD133^{+ve}/CD34^{+ve}$ cells (n=40, R=-0.01, P=0.95 and R=-0.001, P=0.99), eCFU number (n=38, R=0.08, P=0.64 and R=0.06, P=0.73), HPC migration capacity (n=29, R=0.06, P=0.77 and R=0.07, P=0.72) or HPC senescence (n= 35, R=0.15, P=0.38 and R=0.17, P=0.33) all analysed using Spearman's rank correlation). There were no significant relationships between other clinical parameters (such as co-morbidities) and operative parameters (such as the use of a patch) and HPC number or function.

3.3 Circulating $CD133^{+ve}/CD34^{+ve}$ cell number following endarterectomy

Circulating $CD133^{+ve}$ /$CD34^{+ve}$ cells (per 200,000 PBMNC) were measured by flow cytometry in 62 patients undergoing CEA pre-operatively, in 61 patients post-operatively and in 34 patients at 6 weeks post-operatively. There was a significant reduction in circulating $CD133^{+ve}$ /$CD34^{+ve}$ cell numbers 1 day post-operatively, with no significant change from 1 day post-operatively to 6 weeks post-operatively ($P<0.001$, Friedmans Test, pre-op vs 24hr post-op, $P<0.05$, 24hr post-op vs 6wks post-op, $P>0.05$, and pre-op vs 6 weeks post-op, $P<0.05$, Dunn's post test, Table 13 and Fig 31). Analysis of $CD133^{+ve}$ and $CD34^{+ve}$ individual cell populations revealed a similar pattern (Table 13 and Fig 32, 33).

The post-operative changes varied greatly between patients. $CD133^{+ve}$ /$CD34^{+ve}$ cell numbers fell 24hrs post-operatively by a median of 28%, with a range from a fall of 75% to a rise of 50% (Fig 35). The change from postoperative levels to 6 week levels were also highly variable. The median rise was 28% with the range from a fall of 70% to a rise of 200%.

Table 13. Changes in $CD133^{+ve}/CD34^{+ve}$, $CD133^{+ve}$ and $CD34^{+ve}$ cell populations pre-operatively, 24hr and 6 weeks post-operatively (number of cells/200,000 PBMNCs; * Friedman's test).

Cell type	Pre-op (n=62) median(range)	Post-op (n=61) median(range)	6 weeks (n=34) median(range)	P-value*
$CD133^{+ve}/CD34^{+ve}$	111(32-508)	82 (8-293)	73 (24 - 436)	<0.001
$CD133^{+ve}$	128(35-527)	99(25-326)	102(29-554)	<0.001
$CD34^{+ve}$	151(33-594)	122(10-413)	86(15-455)	<0.001

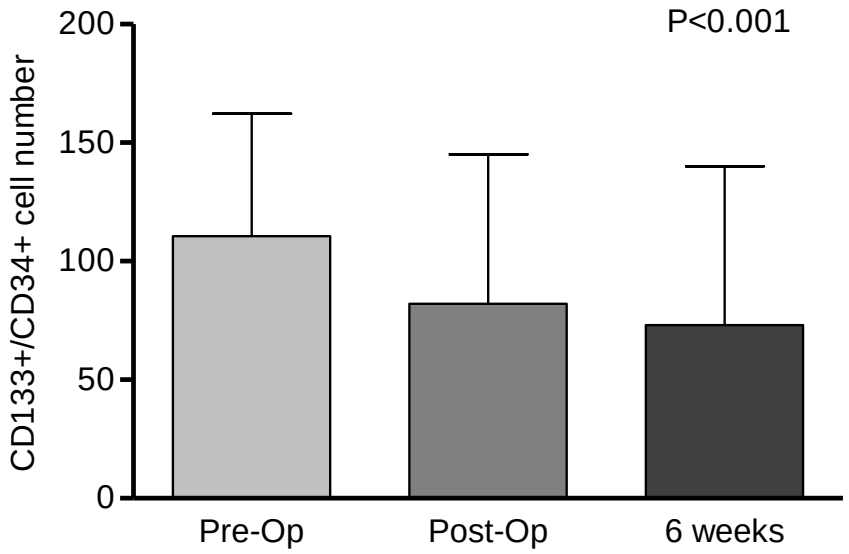


Fig 31. Changes in the number of PBMNCs expressing both CD133 and CD34 pre-operatively, 24hr and 6 weeks post-operatively (Bars=interquartile range, Friedman's Test)

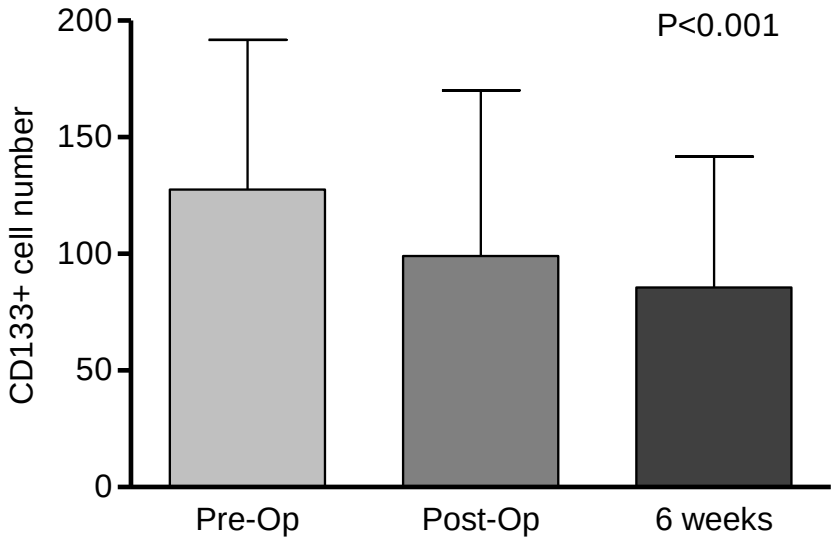


Fig 32. Changes in the number of PBMNCs expressing CD133 pre, 24hr post and 6 weeks post-operatively (Bars=interquartile range, Friedman's Test)

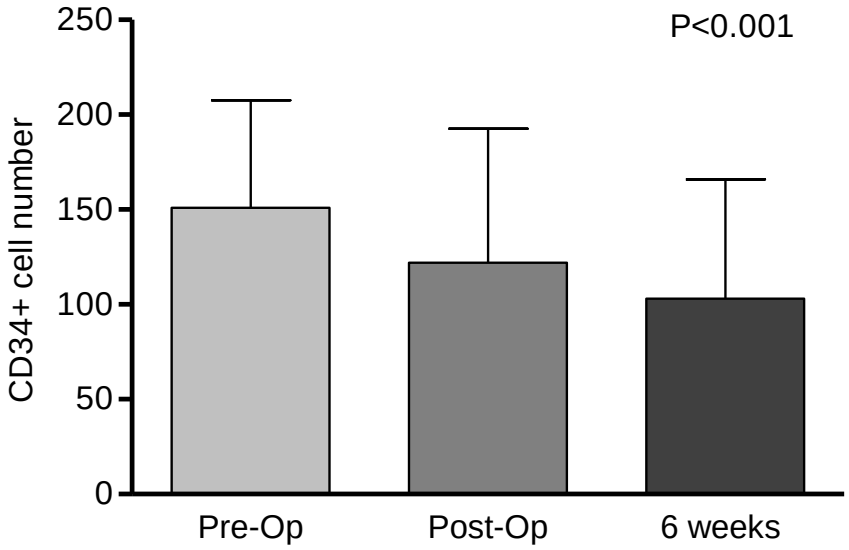


Fig 33. Changes in the number of PBMNCs expressing CD34 pre, 24hr and 6 weeks post-operatively (Bars=interquartile range, Friedman's Test)

3.4 eCFU assay

HPC number was measured by eCFU assay in 58 patients pre-operatively, in 57 patients post-operatively and in 33 patients at 6 weeks post-operatively. There was a significant reduction in eCFU numbers (mean [±SD]) 1 day post-operatively followed by a rise to pre-operative levels by 6weeks post-operatively (16(±3.5), 13(±3.5) and (16(±3), pre-op, 1 day post op and 6 weeks post-op respectively, P=<0.001, ANOVA, pre-op vs 24hr post-op, P<0.05, 24hr post-op vs 6wks post-op, P<0.05, and pre-op vs 6wks post-op, P>0.05, Bonferroni's post test, Fig 34). The post–operative change in eCFU number varied greatly between individuals (similar to that seen for circulating $CD133^{+ve}/CD34^{+ve}$ cells). The mean(±SD) percentage fall from pre to post-operative levels was 17.4 (±14)% with a range varying from a fall of 50% to a rise of 9%. The mean rise from post-operative to 6 weeks levels was 29(±27)% with a range varying from a fall of 13% to a rise of 125%. The variation in the percentage change from preoperative to postoperative levels in $CD133^{+ve}/CD34^{+ve}$ cells and eCFUs can be seen in Fig 35.

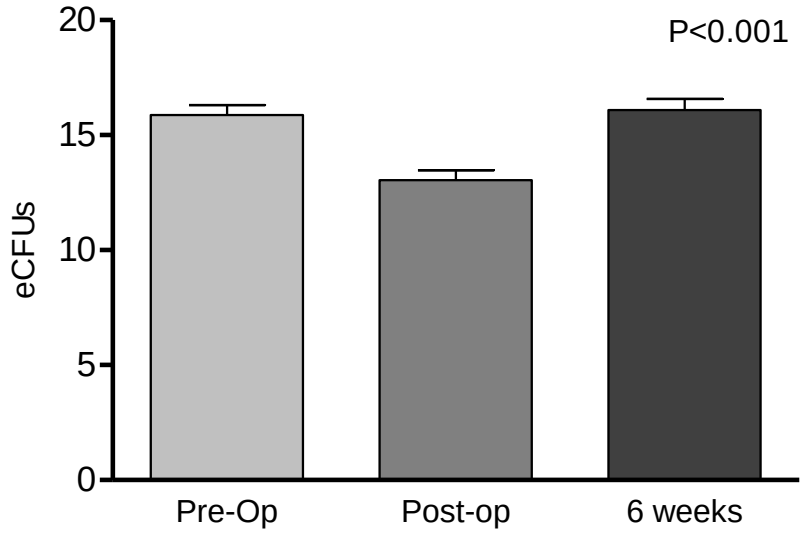


Fig 34. Change in CFU number from pre-op to 6 weeks post-op (Bars=SEM, ANOVA).

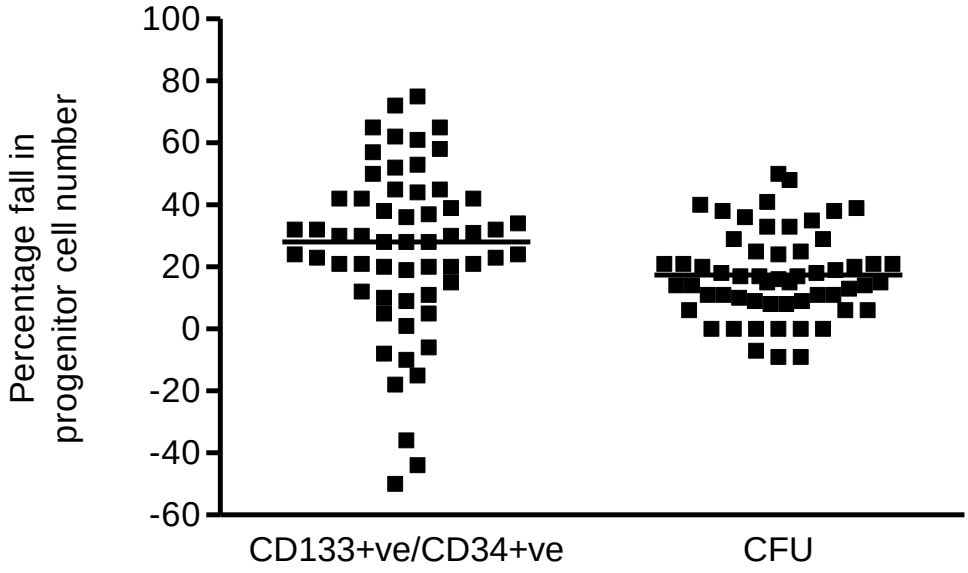


Fig 35. Distribution in the percentage fall of $CD133^{+ve}/CD34^{+ve}$ cell and eCFUs number from pre to 24hour post-operative levels (bars=mean).

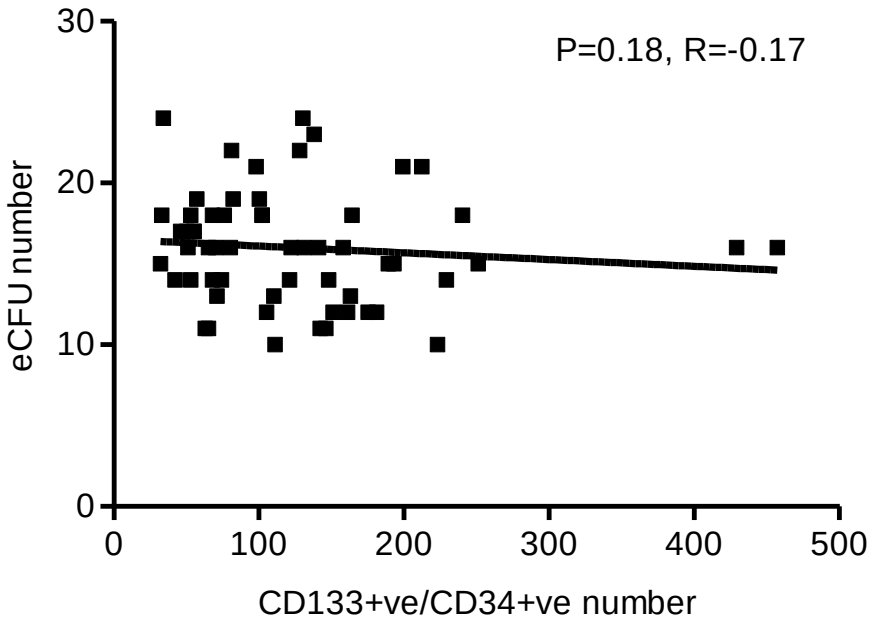


Fig 36. Correlation of pre-operative CD133^{+ve} /CD34^{+ve} number with eCFU number (Spearman's correlation).

The similar pattern of change in post-operative CD133^{+ve}/CD34^{+ve} cell number and eCFU number led us to investigate the relationship between the two variables. There was no direct correlation between CD133^{+ve}/CD34^{+ve} cell number and eCFU number (R=-0.17, P=0.18, Fig 36).

3.5 HPC migration

HPC migration was measured in 47 patients pre-operatively, in 39 patients post – operatively and a further 24 of these also had measurements at 6 weeks. The migration capacity of HPCs was expressed as the percentage increase in the mean fluorescence intensity at 4hrs in response to VEGF solution compared with

vehicle control (baseline). There was no significant change in the HPC migration capacity post-operatively or at 6 weeks (P=0.72 ANOVA, P>0.05 for all group comparisons - Bonferroni's post test, Table 14).

Table 14. Migration of HPCs isolated from the blood of patients undergoing CEA pre, post and 6 weeks post-operatively (P=0.72, ANOVA).

HPC migration	Mean fluorescence intensity above baseline	SD
Pre-operative	19.7	10.2
1 day post-operative	20.2	9.4
6 weeks post-operative	21.7	10

3.6 HPC senescence

HPC senescence was measured in 54 patients pre operatively, in 46 patients post-operatively and a further 26 of these also had measurements at 6 weeks. The mean (±SD) pre-operative progenitor cell senescence was 26.2 (±4.2)%. There was no significant difference in the HPC senescence measured pre-operatively, post-operatively and 6 weeks post-operatively (P=0.7 - ANOVA, P>0.05 for all group comparisons - Bonferroni's post test Table 15).

Table 15. Senescence of HPCs isolated from the blood of patients undergoing CEA pre, post and 6 weeks post-operatively (P=0.7, ANOVA).

HPC senescence	% Senescence (mean)	SD
Pre-operative	26.2	4.2
1 day post-operative	25.6	5.2
6 weeks post-operative	26.8	3.5

3.7 The relationship between restenosis and HPC number and function

3.7.1 Restenosis after CEA

Postoperatively, 51 patients attended for the 3 month scan, 44 for the 6 month scan, and 24 for the 12 month scan. There was a significant progression in the degree of ICA stenosis over this period (median(range) 3 months – 16(15-70)%, 6 months - 30(15-90)%, 12 months - 40(16-80)%, $P<0.001$, Kruskal Wallis, Fig 37). Post test (Dunns) showed that there was a significant change ($P<0.05$) from 3 to 6 months and from 3 to 12 months but not from 6 to 12 months ($P>0.05$).

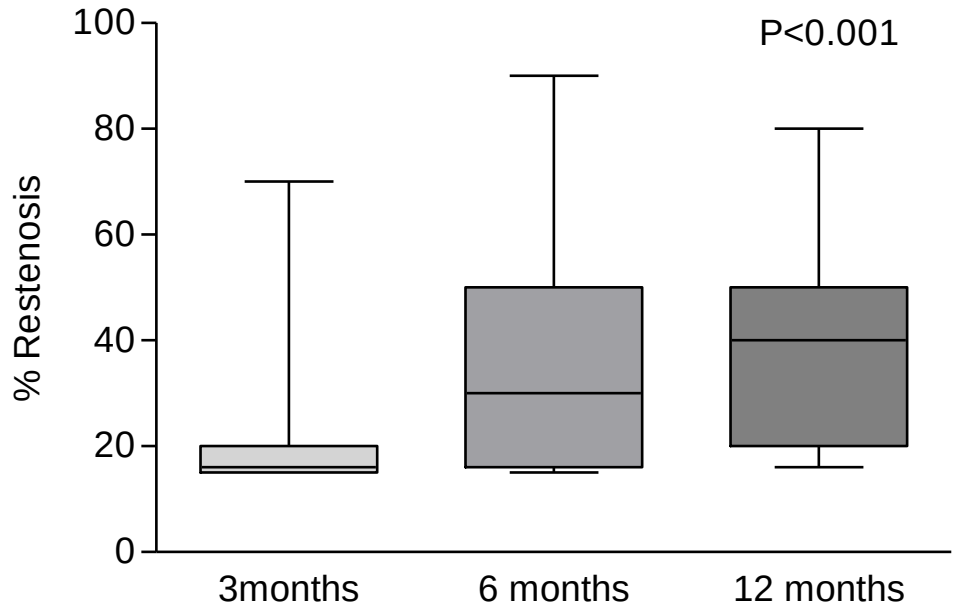


Fig 37. Percentage restenosis following CEA as measured by Duplex ultrasonography at 3 (n=51), 6 (n=44) and 12 (n=24) months post-operatively (Bars = median, Kruskal Wallis test)

3.7.2 Pre-operative circulating HPC number and restenosis

Significant restenosis (i.e. ≥50%) was seen in 6 out of 51 (11%) patients at 3 months, 15 out of 44 (33%) patients at 6 months and 8 out of 24 (33%) patients at 12months. There was no significant difference in the number of pre-operative circulating $CD133^{+ve}/CD34^{+ve}$ cells in patients who developed <50% restenosis compared with those that developed >50% restenosis (Table 16) at the 3month scan (P=0.5, Mann Whitney U test). This was also the case for restenosis at 6 month (P=0.43, Fig 38); and at 12 month scans (P=0.27, all Mann Whitney U test). This relationship was also observed for pre-operative eCFU numbers (Fig 39) and

restenosis at the 3, 6 and 12month scans (Table 16) and for 1 day post-operative and 6 weeks postoperative counts.

Table 16. Pre-operative $CD133^{+ve}/CD34^{+ve}$ cell and eCFU counts in patients with restenosis <50% compared with ≥50% at the 3, 6 and 12 months scans. (# Mann Whiney U test; *Students t-test)

Pre-operatively isolated cells	Post-op scan time (months)	<50% restenosis	≥50% restenosis	P value
$CD133^{+ve}$ /$CD34^{+ve}$ cells numbers (per $2x10^5$ PBMNC) (median(range))	3	102(33-508) n= 43	93.5 (34-457) n= 6	0.5#
	6	102 (32-429) n=27	122(34-508) n=15	0.43#
	12	80(46-429) n= 15	150(55-508) n=8	0.27#
eCFU number (mean(±SD))	3	16 (±3) n= 40	18 (±5) n=6	0.16*
	6	16(±3) n= 27	18(±4) n=12	0.07*
	12	15(±5) n= 14	17(±4) n=6	0.08*

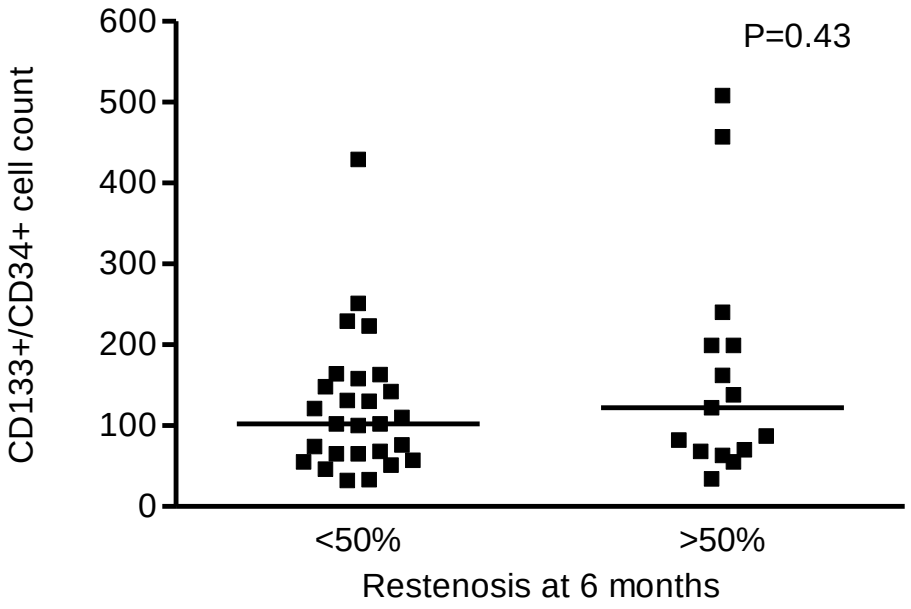


Fig 38. Pre-operative CD133+ve/CD34+ve cell count in patients with restenosis at 6 months (Bars = medians, Mann Whitney U test).

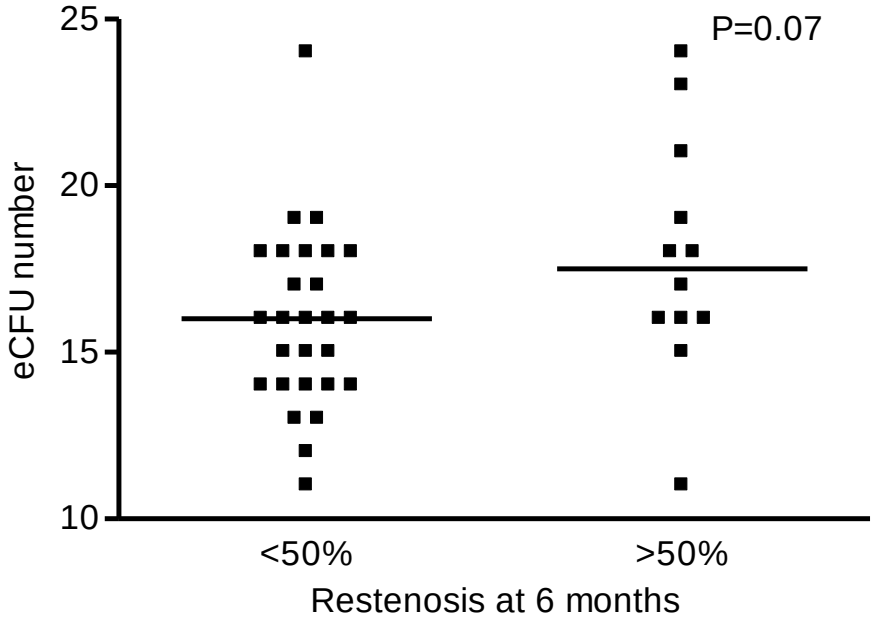


Fig 39. Pre-operative eCFU count in patients with restenosis at 6 months (Bars = means, t-test).

3.7.3 Post-operative change in HPC number and restenosis

Patients who developed a restenosis of <50% had a significantly greater post-operative fall in $CD133^{+ve}/CD34^{+ve}$ cells when compared with those that developed restenosis of ≥50% at the 6 month scan (P=0.044, Table 17, Fig 40), but not at the 3 month or 12 month scans (P=0.66 and P=0.95 respectively, all Mann Whitney U test, Table 17). Patients with a restenosis of <50% at the 6 and 12month scans also had significantly greater falls in eCFUs post-operatively when compared with those who had ≥50% restenosis at these times (P=0.003, Fig 41 and P=0.03, all t-test, Table 17). However this difference was not present when comparing the two groups (<50% vs ≥50% restenosis) on the 3month scans (P=0.34, t-test)

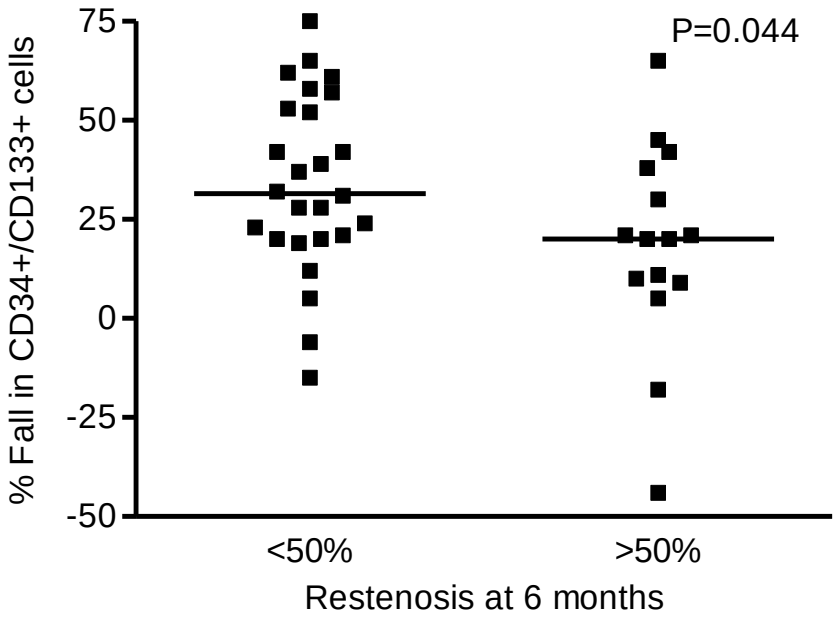


Fig 40. The post-operative percentage fall in $CD133^{+ve}$ /$CD34^{+ve}$ cells in patients developing restenosis at 6months (Mann Whitney U test).

Table 17. Percentage fall in $CD133^{+ve}/CD34^{+ve}$ cell and eCFU numbers 24hr post-operatively in patients with restenosis <50% compared with ≥50% at the 3, 6 and 12 months scan (# Mann Whitney U test; *Students t-test)

Post-op scan time (months)		Post-operative fall (%)		P value
		<50% restenosis	≥50% restenosis	
$CD133^{+ve}/CD34^{+ve}$ cells (median&range)	3	27.9(-44-65) n=42	20.2(-44-62) n=6	0.66#
	6	31.5(-15-75) n=26	20.5(-44-65) n=15	**0.044**#
	12	26.5 (-18-65) n=15	22.5(-45-62) n=8	0.95#
eCFU (mean±SD)	3	15(±12) n=39	20(±15) n=6	0.34*
	6	22(±11) n=26	9.5 (±19.5), n=12	**0.003***
	12	23.7(±16.4) n=14	10.7(±7.9) n=6	**0.03***

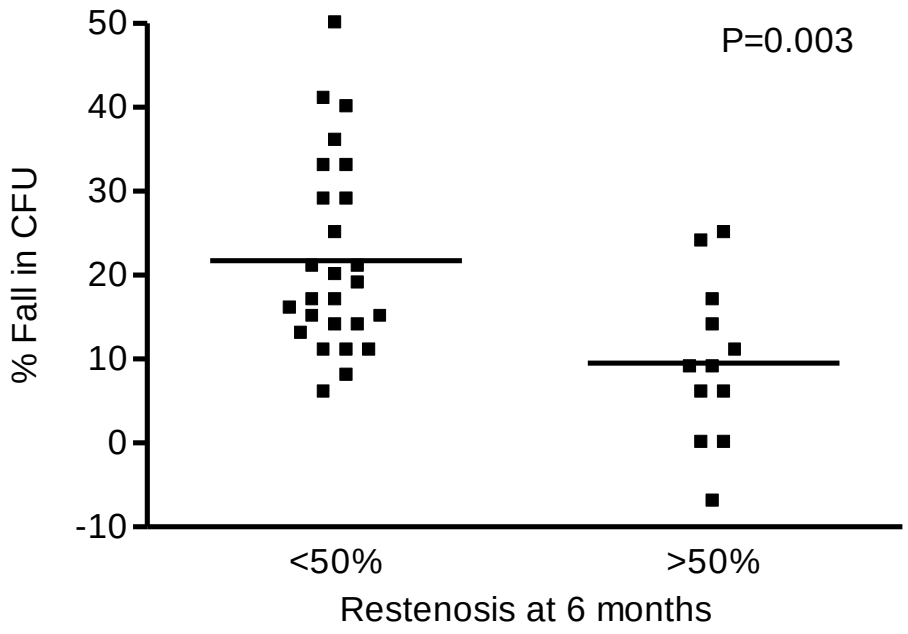
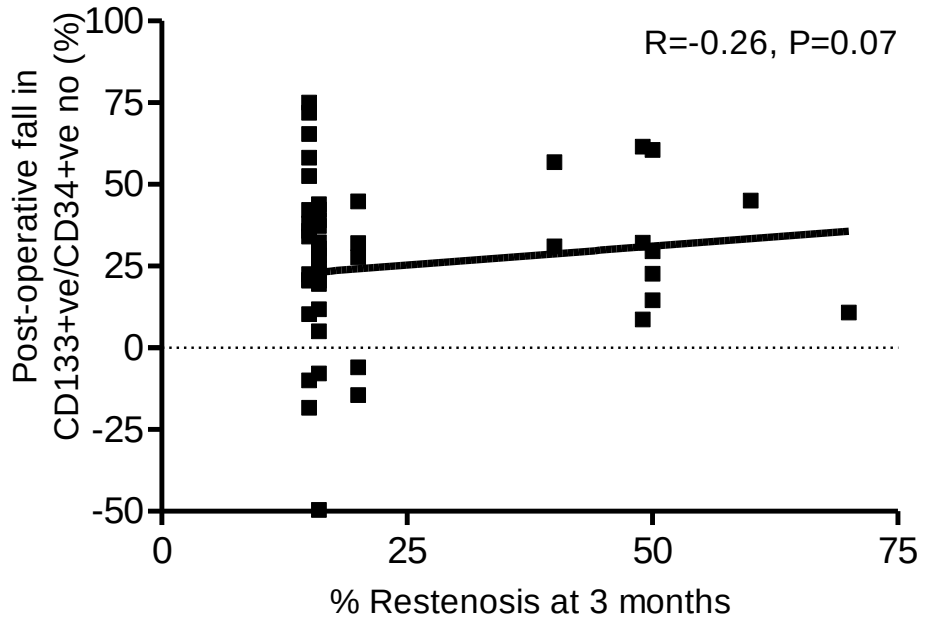


Fig 41. The post-operative percentage fall in eCFU number in patients developing restenosis at 6months (t-test)

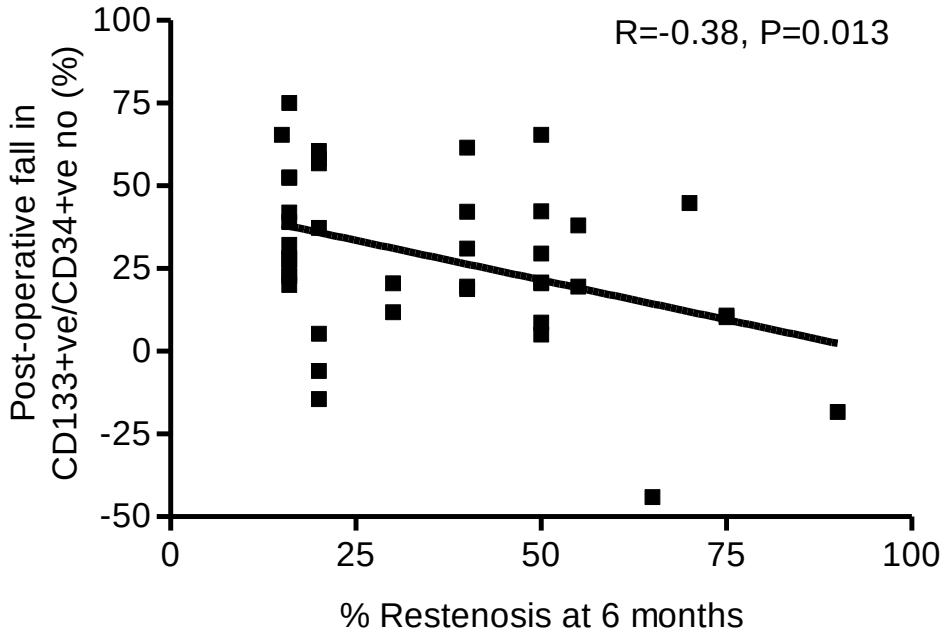
When restenosis was analysed as a continuous variable, there was a negative correlation between the percentage fall in circulating $CD133^{+ve}/CD34^{+ve}$ cell numbers post-operatively and the degree of restenosis at 6months (P=0.013, Table18), with greater falls associated with a smaller degree of restenosis (Fig.42B). There were no significant correlations between these variables at 3 and 12months (Fig 42A and Fig42C). The post-operative fall in CFU number also correlated negatively with degree of restenosis at 6 (P=0.008, Fig 43B) and 12months (P=0.026, Fig 43C), but not 3months (P=0.66, Fig 43A, Table18).

Table 18 Association between the post-operative change in HPC number and function and restenosis as a continuous variable at 3, 6 and 12 months (R= Spearman's correlation coefficient, N = number of samples)

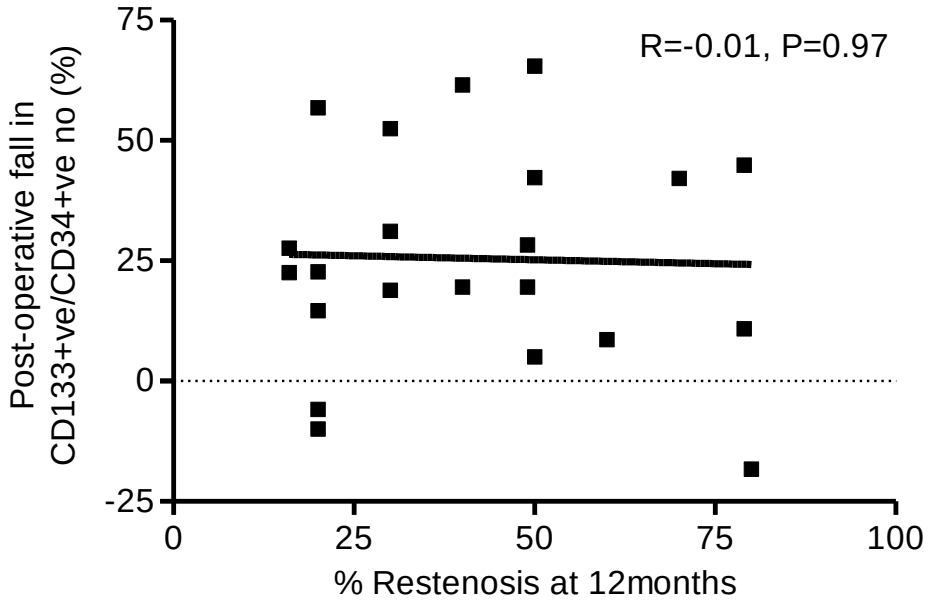
		Restenosis Duplex Scan Time (months)		
		3	6	12
$CD133^{+ve}/CD34^{+ve}$ post-op change (%)	R	-0.26	-0.38	-0.01
	P value	0.071	**0.013**	0.97
	N	48	41	23
eCFU post-op change (%)	R	-0.07	-0.42	-0.49
	P value	0.66	**0.008**	**0.026**
	N	45	38	20
HPC Migration	R	-0.22	-0.48	-0.53
	P value	0.172	**0.007**	**0.05**
	N	39	31	14
HPC Senescence	R	0.27	0.33	0.10
	P value	0.081	**0.05**	0.70
	N	43	37	16


R=-0.26, P=0.07
Post-operative fall in CD133+ve/CD34+ve no (%)
100
75
50
25
0
-25
-50
0
25
50
75
% Restenosis at 3 months

A

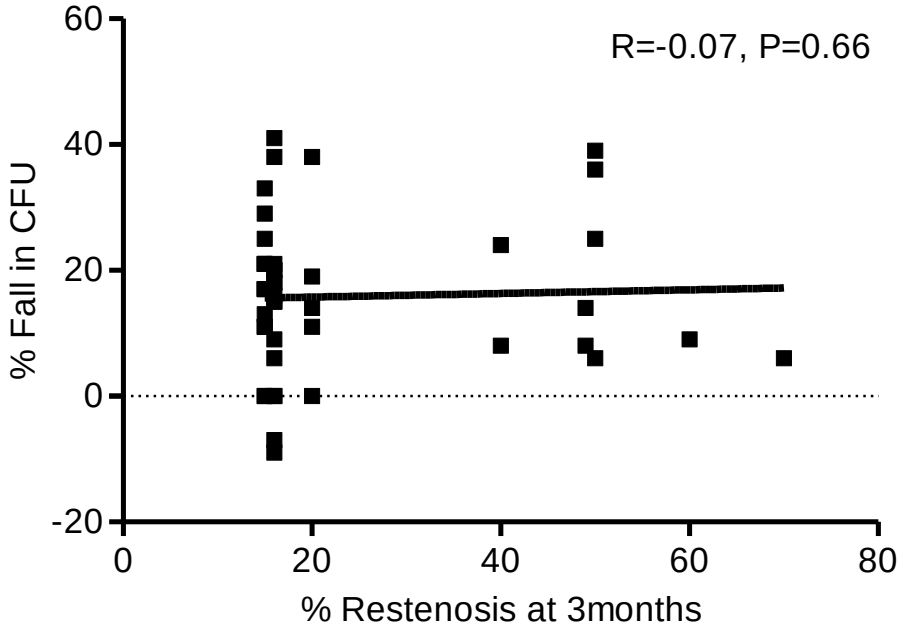

R=-0.38, P=0.013
Post-operative fall in CD133+ve/CD34+ve no (%)
100
75
50
25
0
-25
-50
0
25
50
75
100
% Restenosis at 6 months

B

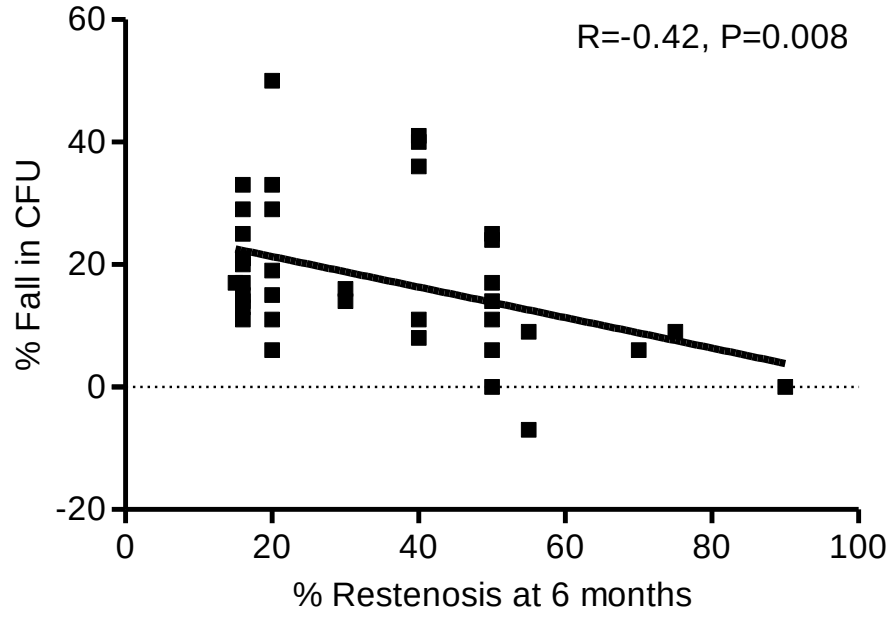


c

Fig 42. The relationship between post-operative percentage fall in CD133^{+ve} /CD34^{+ve} cell number and restenosis as a continuous variable at: A) 3 months: B) 6 months; C) 12 months (R=Spearman's Rank Correlation)



A



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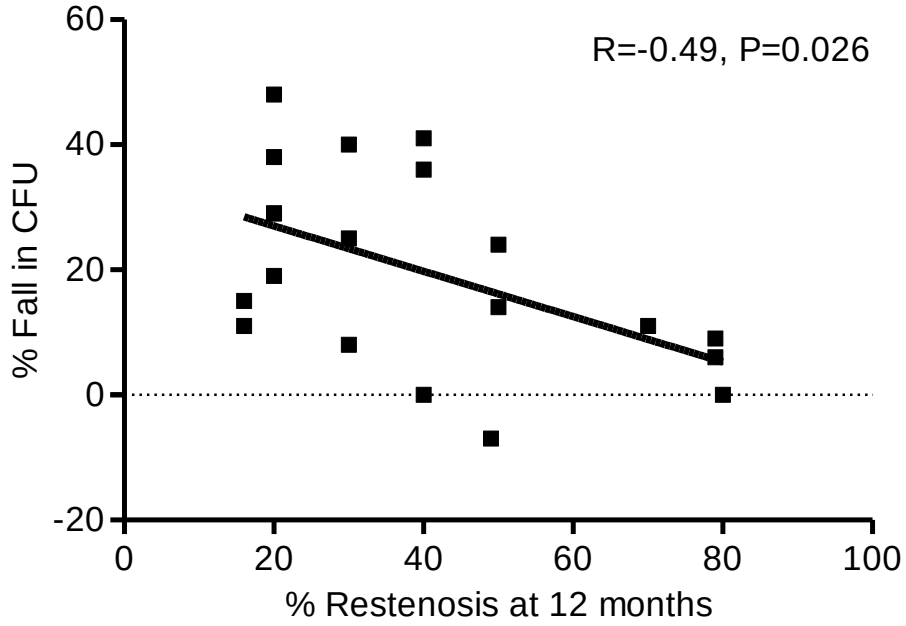


C

Fig 43. The relationship between post-operative fall in HPC-CFU number and restenosis as a continuous variable at: A) 3 months; B) 6 months; C) 12 months (R=Spearman's Rank Correlation)

Table 19. Percentage rise in $CD133^{+ve}/CD34^{+ve}$ cell and eCFU numbers 1 day to 6 weeks post-operatively in patients with restenosis <50% compared with ≥50% at the 3, 6 and 12 months scan. (# Mann Whitney U test; *Students t-test)

Post-op scan time (months)		Post-operative rise (%)		P value
		<50% restenosis	≥50% restenosis	
$CD133^{+ve}/CD34^{+ve}$ cells (median&range)	3	27(-69-198) n=25	20.2(-45-100) n=3	0.91#
	6	38(-51-200) n=21	19(-45-174) n=6	064#
	12	32 (-36-128) n=10	74(9-174) n=4	0.32#
eCFU (mean±SD)	3	24(±12) n=24	34(±30) n=3	0.6*
	6	34(±30) n=20	14 (±15) n=6	**0.04***
	12	34(±29) n=10	13(±11) n=4	0.06*

There was no significant change in $CD133^{+ve}/CD34^{+ve}$ cell number from 1 day post-operatively to 6 weeks post-operatively (section3.4). There was no significant relationship between the magnitude of this change and restenosis at 3, 6 or 12 months (Table 19). Patients with a restenosis of <50% at the 6 month scan had a significantly greater rise in eCFUs from 1 day to 6 weeks post-operatively when compared with those who had ≥50% restenosis (P=0.04 t-test, Table 19) mirroring the relationship observed between restenosis and the post-operative fall in eCFU numbers. This relationship was not present when comparing the two groups (<50% vs ≥50% restenosis) on the 3month (P=0.6) and 12 month scans (P=0.06, both t-test).

3.7.4 HPC function

Preoperative HPC migration was significantly higher in patients who had <50% restenosis at 6 and 12 months compared with those that had >50% restenosis (P=0.01, Fig. 44 and P=0.02 respectively, t-test, Table 20). There was no difference in HPC migration at 3months (P=0.45) HPC migration correlated negatively with restenosis as a continuous variable (Table 18) at 6 months (R=-0.48, P=0.007, Fig. 45B) and 12months (R=-0.53, P=0.05, all Spearman's Rank Correlation, Fig 45C). There was no correlation between HPC migration and degree of restenosis at 3months (P=0.17, Fig 45A).

There was no association between HPC senescence and restenosis (<50% vs ≥50%) at the 3, 6 or 12months (P= 0.06, P=0.6 and P=0.6 respectively, all t-test,

Table 20). There was no significant correlation between HPC senescence and restenosis as a continuous variable at any of the scan times (Table18).

Table 20. HPC migration and senescence of pre-operatively isolated cells from patients who developed restenosis <50% compared with those who developed ≥50% restenosis at 3, 6 and 12 months (t-test).

End point measured	Scan time (months)	Restenosis		P-value *
		<50%	≥50%	
HPC migration (mean±SD)	3	19(±11) n=33	22(±8) n=6	0.45
	6	24(±11) n= 17	15(±6) n=9	**0.01**
	12	22(±7) n=7	13(±4) n= 5	**0.02**
Senescence (mean ±SD)	3	27(±4) n= 37	29(±3) n=6	0.06
	6	26(±4)% n=25	27(±5.0)% n=12	0.6
	12	27(±3)% n= 10	26(±3)% n=6	0.6

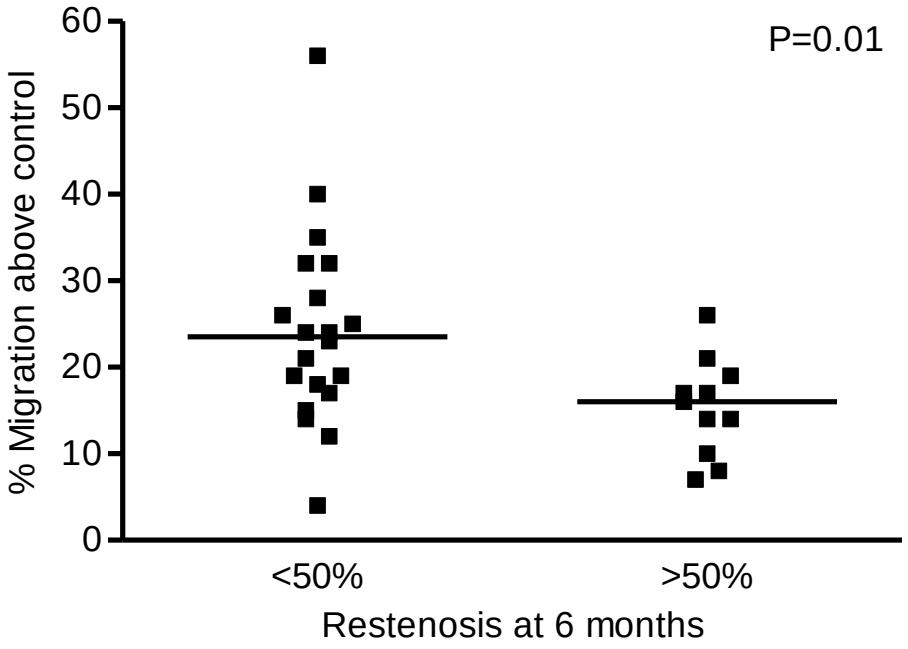
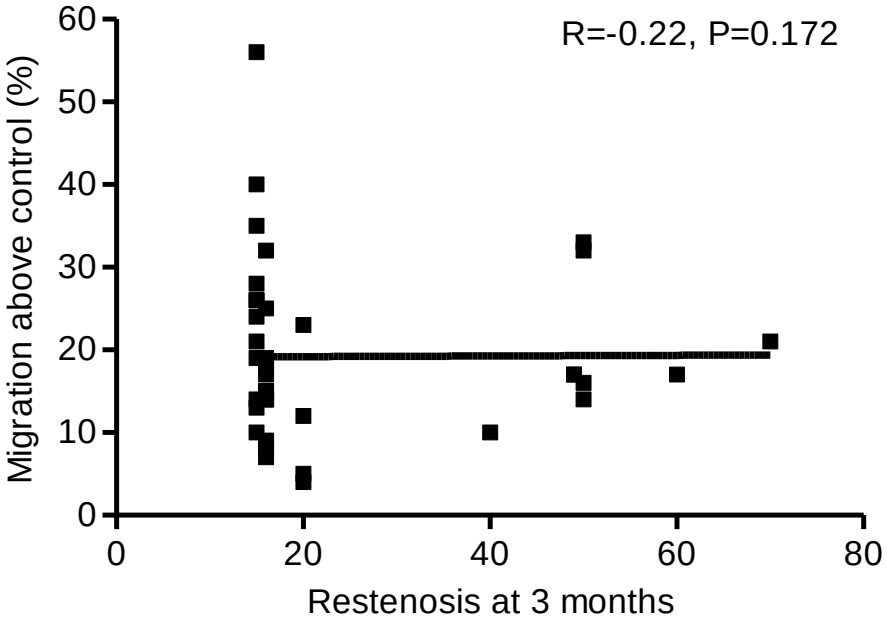
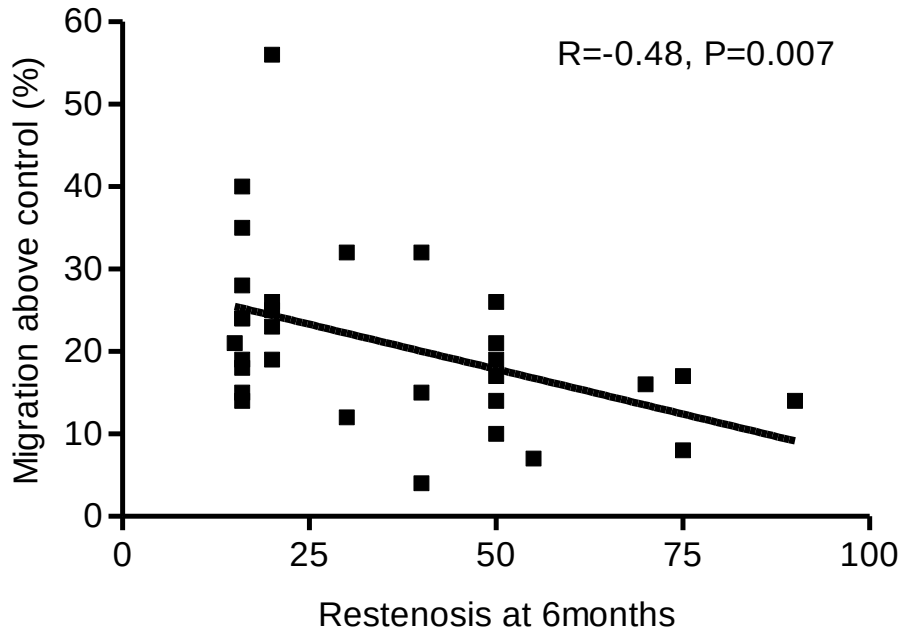


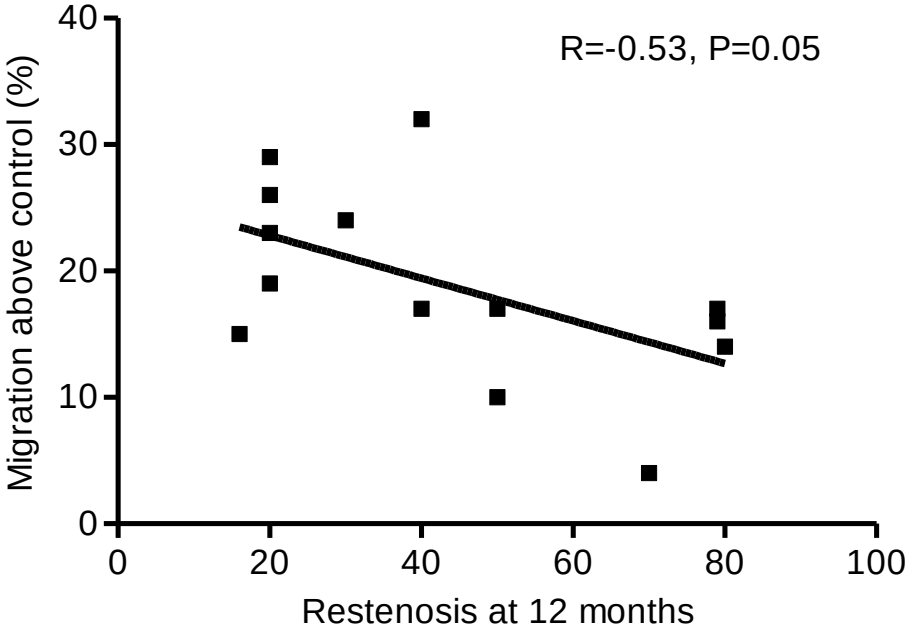
Fig 44. Pre-operative HPC migratory capacity in those patients with a restenosis of <50% and >50% at 6 months (t-test).



A



B



C

Fig 45. The relationship between pre-operative HPC migration and restenosis as a continuous variable at: A) 3 months; B) 6 months; C) 12 months (R=Spearman's rank correlation).

3.8 Cytokine levels and restenosis

The circulating levels of cytokines known to be involved in HPC mobilisation and chemotaxis were measured in the cohort of patients that had the full set of HPC investigations at all three time points (i.e. pre-, 24hrs and 6 weeks post-operatively, n=24, Table 21). GM-CSF and PlGF levels fell 24hrs post-operatively before rising at 6wks, however these changes were not significant (P=0.47 and P=0.46 respectively, ANOVA, Fig 46). SDF-1 levels fell significantly post-operatively and rose to pre-operative level by 6weeks (P=0.003 Repeated measures ANOVA, Fig 46, P<0.05 – pre-op vs 24hr post-op and P<0.05 - 24hr post-op vs 6wks post-op, P>0.05 – pre-op vs 6wks post-op, Bonferroni's post test) The circulating levels of VEGF increased 24hrs post-operatively and continued to rise by 6weeks (P=0.028, Repeated measures ANOVA, P>0.05 – pre-op vs 24hr post-op, P>0.05 - 24hr post-op vs 6wks post-op, P<0.05 – pre-op vs 6wks post-op, Bonferroni's post test, Fig 46). There was no association between any of the cytokine levels and the degree of restenosis when analysed as either a binary end point (i.e <50% vs ≥50%) or as a continuous variable (Table 22).

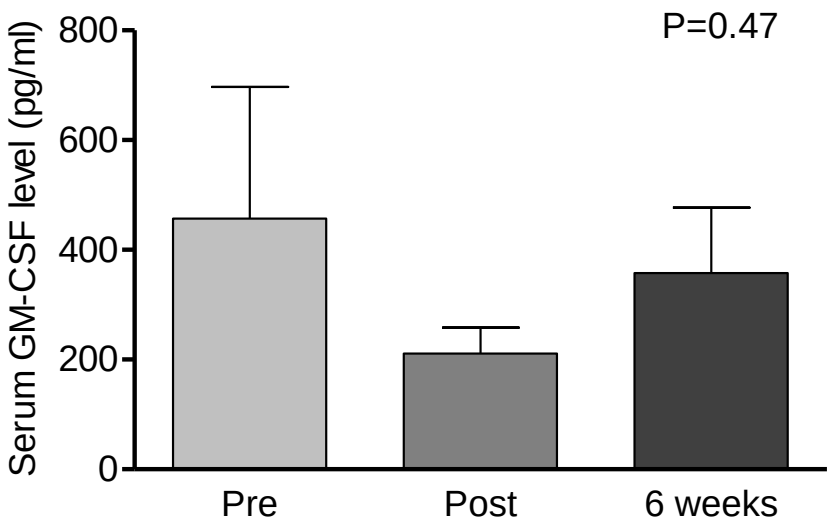
Pre-operative SDF-1 levels correlated with circulating $CD133^{+ve}/CD34^{+ve}$ cell numbers pre-operatively (R=-0.46, P=0.025, Fig 47) and both the post-operative percentage change in $CD133^{+ve}$ /$CD34^{+ve}$ numbers and percentage change in CFU number (R=0.42, P=0.04, Fig 48 and R=0.56, P=0.004, Fig 49, respectively, both Spearman's Rank Correlation, Table 23). However there was no significant

correlation between these variables and the levels of GM-CSF, PlGF and VEGF (Table 23).

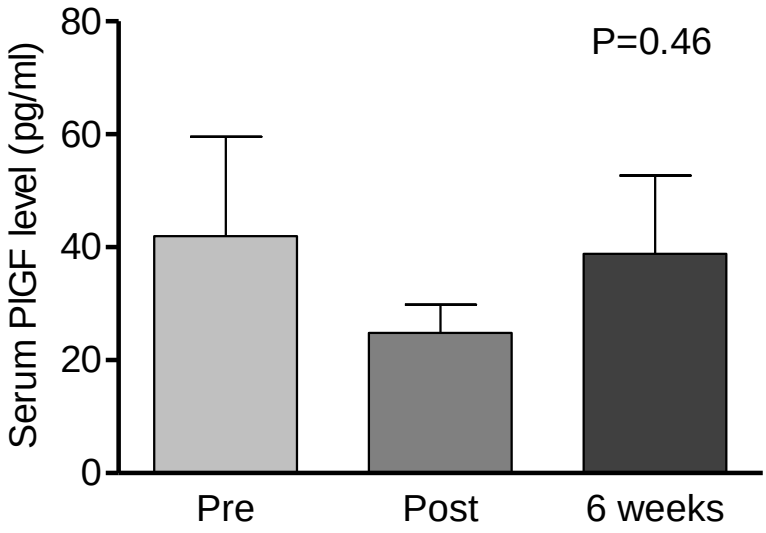
Table 21. Serum concentrations of GM-CSF, PlGF, SDF-1 and VEGF pre, post and 6 weeks post-operatively (* Repeated measures ANOVA).

Cytokine	Cytokine concentration (pg/ml) (mean±SD)			P-value *
	Pre-operative	1 day post-operative	6 weeks post-operative	
GM-CSF	458(±1177)	211(±232)	358(±585)	0.47
PlGF	42(±86)	25(±24)	39(±68)	0.46
SDF-1	951(±312)	763(±270)	924(±262)	**0.003**
VEGF	43(±51)	52(±70)	112(±160)	**0.028**

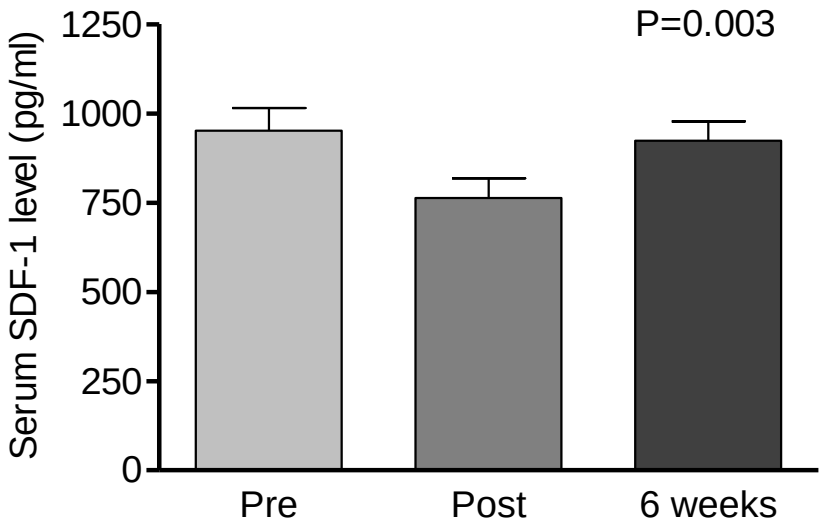
A


P=0.47
Serum GM-CSF level (pg/ml)
800
600
400
200
0
Pre
Post
6 weeks

B


P=0.46
Serum PlGF level (pg/ml)
80
60
40
20
0
Pre
Post
6 weeks

C



D

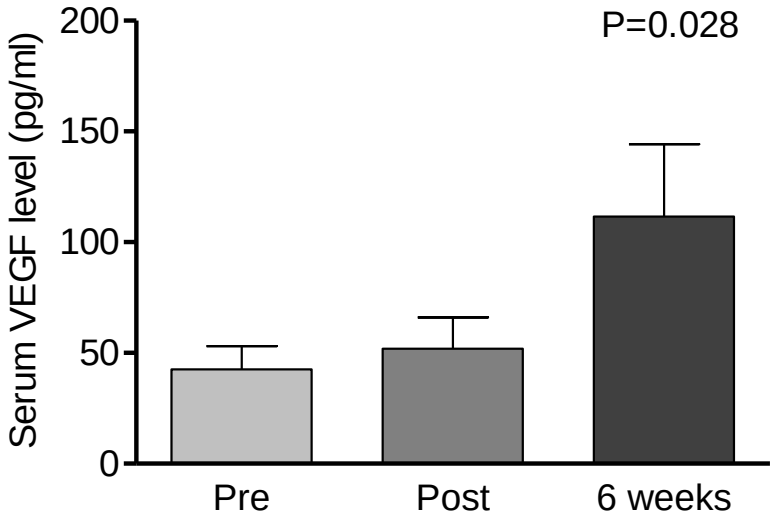


Fig 46. Serum concentrations of (A) GM-CSF; (B) PlGF; (C) SDF-1; (D) VEGF; Pre-operatively (pre), 24 hrs post-operatively (post) and 6 weeks post-operatively (6 weeks)(ANOVA).

Table 22. The correlation between pre-operative serum concentrations of GM-CSF, PlGF, SDF-1 and VEGF and restenosis as a continuous variable at 3, 6 and 12 months (R=Spearman's rank correlation).

Cytokine (pg/ml)		Restenosis 3m	Restenosis 6m	Restenosis 12m
GMCSF	R value	-0.18	-0.14	-0.29
	P value	0.43	0.56	0.42
	N	22	19	10
PlGF	R value	-0.401	-0.19	-0.50
	P value	0.06	0.43	0.15
	N	22	19	10
SDF	R value	0.18	0.22	0.35
	P value	0.42	0.37	0.32
	N	22	19	10
VEGF	R value	0.18	0.16	-0.07
	P value	0.43	0.51	0.84
	N	22	19	10

Table 23. The correlation between serum concentrations of GM-CSF, PlGF, SDF-1 and VEGF and the post-operative percentage change in eCFU and $CD133^{+ve}$ /$CD34^{+ve}$ cells (R=Spearman's rank correlation).

Cytokine (pg/ml)		% Change in eCFU	% Change in $CD133^{+ve}$ /$CD34^{+ve}$ cells
GMCSF pre	R value	-0.06	0.24
	P value	0.77	0.26
	N	24	24
PlGF pre	R value	-0.02	0.18
	P value	0.94	0.41
	N	24	24
SDF pre	R value	**0.56**	**0.42**
	P value	**0.004**	**0.04**
	N	24	24
VEGF pre	R value	-0.05	0.05
	P value	0.82	0.82
	N	24	24

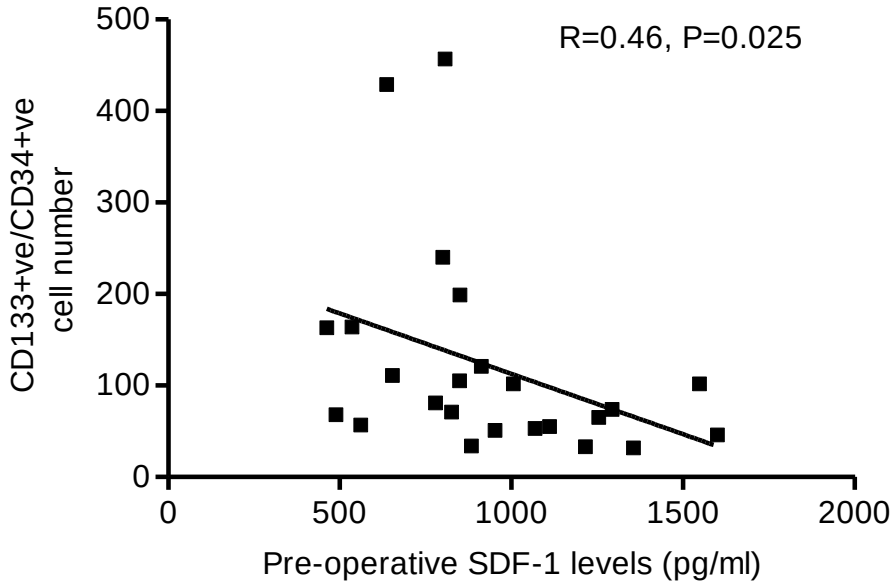


Fig 47. Correlation between pre-operative blood SDF-1 levels and pre-operative circulating CD133+ve /CD34+ve cell numbers (R=Spearman's correlation)

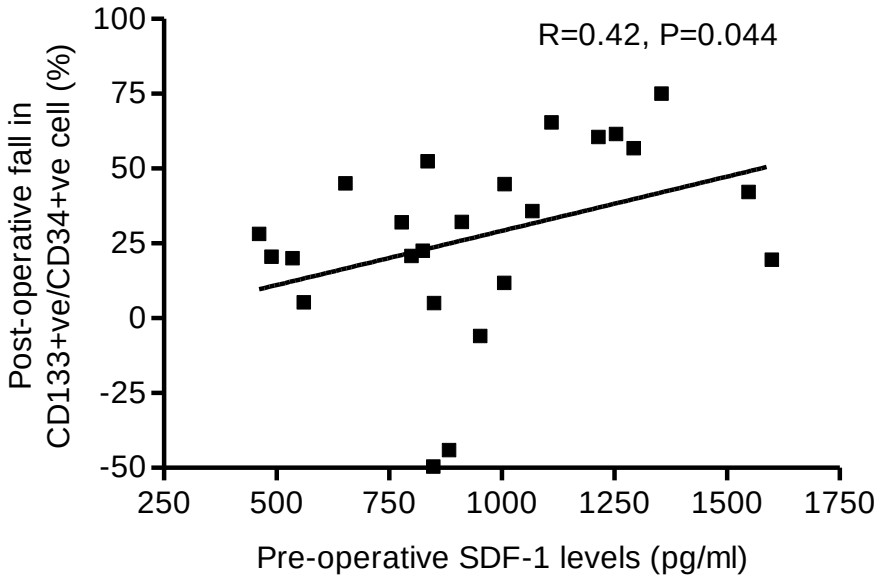


Fig 48. Correlation between pre-operative blood SDF-1 levels and the post-operative percentage fall in CD133^{+ve} /CD34^{+ve} cells (R=Spearman's correlation)

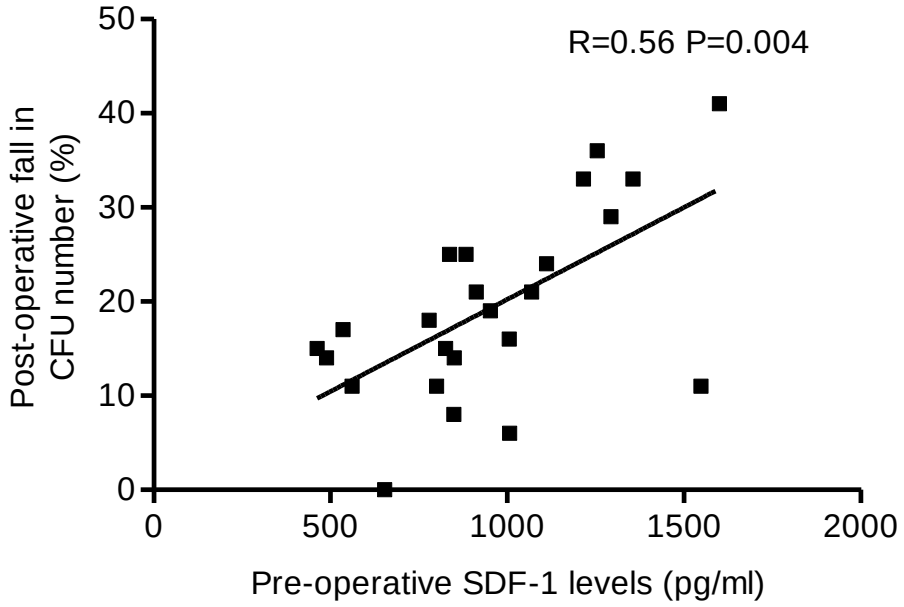


Fig 49. Correlation between pre-operative circulating SDF-1 levels and the post-operative percentage change in eCFU number (R=Spearman's correlation)

3.9 Multivariate analysis

Binary logistic regression analysis was used to look for variables that independently predicted restenosis >50% at 3, 6 and 12 months. All variables that were found to have a significant relationship with restenosis in the univariate analysis were put forward into the multivariate analysis. No factors were found to independently predict restenosis >50%. Further analysis was carried out using linear logistic regression analysis to find factors independently predicting restenosis as a continous variable. Again none of the factors that have been found to be significantly correlated with restenosis on univariate analysis were independant predictor of restenosis on multivariate analysis.

4. DISCUSSION

4.1 Measurement of HPC number and function

Asahara et al[101] first described the out growth of a colony of endothelial cells when CD34 enriched peripheral blood mononuclear cells were grown in culture and expressed the endothelial marker, VEGF receptor-2 (VEGFR2). It was suggested that these cells represented a population of endothelial progenitor cells (EPCs). Over the past 10 years there have been a number of studies that suggest bone marrow-derived EPCs can differentiate into mature endothelial cells (EC) and maintain endothelial function[111, 112]. EPCs have been defined by the expression or co-expression of various combinations of markers including CD34[101], VEGFR2[101] and CD133[113,116], and are thought to have the functional capacity to form colonies (CFUs) in vitro[101, 111, 121]. Early CFUs (i.e. those colonies that form between 5 and 10days in culture) have subsequently been found to express haematopoietic cell surface proteins[128]. Mononuclear cell isolates depleted of $CD34^{+ve}$ cells and comprising myeloid cells, are, however, still able to form these colonies in-vitro[197]. It is now generally accepted that what have previously been called EPCs are likely to represent a heterogeneous group of primitive haematopoietic progenitor cells (HPCs) that express both endothelial and myeloid lineage markers[120, 128, 197].

The results of the present study appear to support this mixed phenotype hypothesis in that they show there is poor correlation between the physical and

functional methods of measuring HPC numbers ($CD133^{+ve}/CD34^{+ve}$ cell numbers by flow cytometry and eCFU count). Others have also shown that flow cytometric analysis using various combinations of antibodies against the markers CD34, CD133 and VEGFR2 did not correlate with each other or with the eCFU assay[134].

In contrast to early CFUs, late CFUs/OECs have a high proliferative capacity, generate vascular tubules in-vivo and in-vitro, and do not express haematopoietic markers [113, 121]. The phenotypic definition of OECs has yet to be established and this limits the use of flow cytometry in measuring this rare cell type. In this study we were unable to refine the methodology for growth of late EPCs/OECs (sections 2.2.3.2 and 2.2.4) to give a reproducible assay and we therefore proceeded with measuring eCFUs only in our patients. The eCFU assay measures both the number and function (ability to form colonies of an apparently endothelial-like phenotype) of HPCs. A combination of eCFU and structural phenotypic analysis by flow cytometry ($CD133^{+ve}/CD34^{+ve}$) has been used by many[251-253] to measure changes in what were thought to be EPCs. Our choice of flow cytometric markers in this study was hampered by difficulties encountered with the VEGFR2 antibody as well as access to a flow cytometer with more than 3 colour capability. The combination of eCFU and flow cytometric enumeration of $CD133^{+ve}/CD34^{+ve}$ cells is, however, likely to represent HPC populations that others have found important in the pathophysiological response to vascular injury[251-253] and that may therefore have a role in the response to endarterectomy.

4.2 HPC number and function and risk of stroke

High circulating HPC numbers are associated with improved endothelial function[111] and a reduced risk of developing cardiovascular disease[111, 117]. Reduced circulating HPC numbers are also associated with a significantly higher incidence of cardiovascular death, unstable angina, and myocardial infarction[143]. HPCs have been shown to mobilise to the site of trauma-induced injury[268]. In the context of carotid disease, HPC numbers are significantly higher in patients following ischaemic stroke than in at-risk control subjects[152], though others have shown reduced numbers when compared with control subjects[269]. A reduced circulating HPC number (measured within 48hours of an ischaemic stroke) is associated with a poorer prognosis[152]. These data led us to investigate the link between HPC number and function and carotid disease. We were unable to demonstrate a relationship between HPC number or function and the 1yr and 5yr risk of further ipsilateral stroke as measured by the Oxford tables. We were also unable to demonstrate any relationship between any other clinical or operative parameters and HPC number and function. One possible explanation may lie in the heterogeneity of our study population, particularly with reference to the time from symptomatic event to endarterectomy which was 7.4(±11.5). This meant that we were measuring HPC number and function at greatly variables times from the symptomatic event, and subsequently trying to correlate this with the risk of further stroke. Our study population was also heterogeneous with regards to other clinical parameters (such as co-morbidities) and operative parameters (such as the use of

a patch or shunt). Subgroup analysis of HPC number and function would have been under powered and as a result was not attempted.

4.3 Post-operative changes in HPC number and function following CEA

CEA led to an acute fall in the number of HPC-eCFUs in the acute post-operative period (1day) followed by a recovery of eCFU numbers to pre-operative levels by 6weeks post-operatively. Levels of $CD133^{+ve}$, $CD34^{+ve}$ and $CD133^{+ve}/CD34^{+ve}$ expressing cells remained depressed following CEA, although there was no significant change in the migratory capacity or senescence of the eCFU-derived HPCs . The acute post-operative fall in HPC numbers following CEA confirms the findings of Stein et al[270] who studied changes in circulating HPC numbers following peripheral (carotid and femoral) endarterectomy. It is in contrast, however, with the rise that occurs in the acute phase following injury caused by coronary artery bypass grafting (CABG) [148, 149] or balloon angioplasty [150, 271] and after ischaemic stroke [152] and myocardial infarction [151]. Angioplasty and coronary bypass could induce some degree of ischaemia that leads to mobilisation of HPC numbers, as coronary angioplasty does not promote mobilisation of progenitors in the absence of myocardial necrosis[272]. During endarterectomy, it is possible that the circle of Willis protects the cerebral circulation from ischaemia and so the main stimulus is that of endothelial damage alone. Endarterectomy also leads to a large area of denuded endothelium, which is much greater than that seen following angioplasty.

We postulate that this may lead to recruitment of HPCs to the site of endarterectomy[138] in an attempt to re-endothelialise this area. In the absence of an ischaemic stimulus, this may overwhelm the capacity to mobilise these cells from the bone marrow within the first 24hrs following surgery, leading to the fall in HPC population numbers. Alternatively it is possible that there may have been a rise in HPC numbers following endarterectomy that occurred either within hours or over a much longer time scale (days). By measuring HPC number at 24hrs we may simply have missed the rise seen following other cardiovascular interventions.

Our inability to detect changes in HPC function may be because our assays were not sensitive enough to detect small changes in function or because function is a property that does not change in the response to acute endothelial injury. This is unlike the situation during chronic diseases such as atherosclerosis or hypertension, where there is a significant change in function[140, 254, 273].

4.4 HPC number and function and restenosis

We report an incidence of restenosis (>50%) of 11% at 3 months, 33% at 6 and 12 months. This is higher than the previously reported incidence of 10-20% in the first year following CEA[29-31]. A high drop out rate was encountered as only 24 of the 62 patients recruited to the study attended the 12 month follow up scan duplex scan and therefore this higher than expected rate of restenosis could be a type-II statistical error caused by the relatively low subject numbers assessed. Our aim

was to recruit sufficient patients to allow our sample population to include 15 patients with restenosis at 6 and 12months. This would be sufficient to give a power of 80-90% to detect a difference between groups with a statistical significance of $p<0.05$. We achieved this at 6months, but not at 12months where the high rate of non-attendance resulted in only 8 patients who had >50% restenosis at this time. This again suggests that analysis of the relationship between HPC number and function and restenosis at 12months is at risk of a type-II error.

There was no association between either the number of the pre-operative $CD133^{+ve}/CD34^{+ve}$ population or the number of HPC-eCFUs and the development of restenosis 3, 6 or 12months after carotid endarterectomy. We also found no evidence that HPC senescence influenced the development of restenosis. This is contrary to patients who developed restenosis following coronary angioplasty, who have a decreased number of eCFUs and increased numbers of senescent cells compared with patients who do not develop restenosis[153]. Multivariate analysis shows that an increased number of senescent HPCs is the only independent factor associated with in-stent restenosis[153]. Patients with angiographically demonstrated, diffuse in-stent restenosis have reduced numbers of HPCs (eCFU assay) when compared with those developing focal restenosis[154].

Increased HPC migratory capacity did appeared to protect against restenosis at both the 6 and 12month time points. This relationship was present when restenosis

was analysed as either an absolute number (i.e <50% vs ≥50%) or as a continuous variable. This is the first study to show that there is a direct relationship between HPC migratory capacity and the development of restenosis. HPC migration and proliferation are correlated with endothelial dysfunction[145], and reduced endothelial function is an independent factor predicting restenosis following coronary angioplasty[71]. Our findings support the notion that progenitor cell function rather than their number is important in maintaining endothelial function and attenuating restenosis.

The variability in the magnitude of the post-operative fall in $CD133^{+ve}/CD34^{+ve}$ and eCFU numbers between patients led us to examine the relationship between these falls and the development of restenosis following CEA. We found that a greater acute post-operative fall in both eCFU and circulating $CD133^{+ve}/CD34^{+ve}$ cell number was associated with reduced restenosis. This relationship existed whether restenosis was analysed as an absolute (<50% vs >50%) or continuous variable. Although a post–operative fall in $CD133^{+ve}/CD34^{+ve}$ HPC number following CEA has previously been demonstrated[270], the results of the present study show that this fall has a significant association with the degree of restenosis that subsequently develops. These data and the association between restenosis and enhanced HPC migratory capacity that we have shown, have led us to speculate that the fall in circulating HPC numbers may reflect the rapid recruitment of HPCs to the site of endarterectomy. A more rapid fall in HPC number represents faster recruitment and re-endothelialisation leading to reduced neointimal hyperplasia.

This notion is also supported by the results of studies that show enhanced re-endothelialisation and decreased neointima formation in animal models of arterial injury following infusion of culture-expanded autologous progenitor cells[138, 157], or following their mobilisation after injury using statins[108, 137], granulocyte colony stimulating factor (G-CSF)[158, 159] or granulocyte macrophage-CSF[107]. Patients who develop restenosis after coronary angioplasty also have a lower circulating number and increased senescence of progenitor cells[100, 154,153]. The findings of the present study provides further evidence to support the hypothesis, generated from both animal [107, 108, 137, 138, 157-159] and other human[100,153,154] studies, that HPC activity inhibits neointimal hyperplasia.

4.5 Cytokine Analysis

The cytokine response that leads to HPC mobilisation and recruitment may be different between interventions that lead to endothelial damage alone, such as endarterectomy, and those that also induce some degree of ischaemia such as CABG. In order to characterise the cytokine response to endarterectomy we measured serum levels of GM-CSF, PlGF, SDF-1α and VEGF pre-operatively, 24hr and 6weeks post-operatively. Of these, only VEGF and SDF-1α levels showed significant changes following CEA. The levels of VEGF rose in the immediate post-operative period and continued doing so at 6weeks. This is similar to the pattern described following coronary artery bypass[149]. The pattern of change for SDF-1α is also similar to that seen following coronary artery bypass[149], with an

immediate post-operative fall followed by a rise at 6weeks. Although the levels of the cytokines measured did not correlate with restenosis, our data suggests that SDF-1 levels may have an important role, as the pre-operative levels of this cytokine were strongly correlated with the post-operative fall in both circulating $CD133^{+ve}/CD34^{+ve}$ cells number and eCFU populations. SDF-1α stimulates progenitor cell mobilisation, growth and differentiation[228]. Interaction with its receptor CXCR4 is thought to be important in regulating progenitor cell survival, cell cycle, and mobilisation[213] and this facilitates progenitor cell recruitment to sites of injury in models of neointimal hyperplasia[238]. SDF-1α binds to platelets at the site of injury, triggers CXCR4- and P-selectin-dependent arrest of progenitor cells on injured arteries [238,239]. Rapid adhesion of platelets to the exposed sub-endothelial surface in injured vessels could provide a very effective mechanism of mobilisation and homing of stem cells to the damaged area as platelets are seen early in the inflammatory response. In vitro studies show that SDF-1α induces HPC and $CD34^{+ve}$ cell migration, and $CD34^{+}$ cell adhesion[240, 241]. Taken together with our earlier results (showing that a greater fall in both $CD133^{+ve}/CD34^{+ve}$ cell number and eCFU number is associated with a reduced incidence of restenosis) our studies provide a possible mechanistic paradigm. Higher pre-operative SDF-1α levels lead to increased SDF-1α driven homing of progenitor cells to the site of injury resulting in a greater post-operative fall in progenitor cell number, enhanced neo-endothelialisation and reduced neointima formation.

4.6 Critique and Future Studies

4.6.1 Patient recruitment

The aim of this study was to recruit sufficient patients over a 2-year period to provide a sample of at least 10-15 restenotic patients. Our power calculation (section 2.7.4) in addition to the approximate rate of restenosis from the literature led us to conclude that between 60-100 patients would be required to give a power of 80-90% to detect a difference if there was one in HPC number and function between those that restenosed and those that did not. Departmental data from previous years showed that around 50 CEAs a year were performed at St Thomas' Hospital. However during the study period this fell to closer to 30 CEAs a year. This led to a total cohort of only 62 patients, and as mentioned earlier (section 4.4) along with the low level of attendance at the 12 month scan led to the possibility of a type II error when looking at restenosis at 12 months.

We are uncertain as to why the number of CEAs has fallen over the study period. This may be related to changing patterns of referral within the region or alternatively the effects of best medical management (section 1.1.3.1) in reducing the incidence of symptomatic carotid artery disease. We became aware of this reduction in the number of CEAs 12 months into the study. We sought to establish collaboration with St George's Hospital and this was agreed, but the Ethics and Research and Development approval took far longer and we ultimately ran out of time.

Further studies will take these experiences into account and although multicentre studies are more difficult to manage (particularly in relation to the rapid handling of blood for subsequent analysis by FACs or for cell culture), this will be essential in order to maximise patient numbers and reduce the chances of a type II error.

4.6.2 Progenitor cells and clinical symptoms

We have demonstrated a possible link between HPC number and function and symptomatic carotid artery stenosis, but this was only a correlation between the two variables and possible mechanisms of this association were not studied. We hypothesise that enhanced HPC number and function could lead to enhanced plaque neovascularisation, instability and therefore embolic symptoms. This hypothesis is difficult to test in animals as although one model of spontaneous plaque rupture has been reported[274] it is far from universally accepted. In humans unstable plaque is available for analysis of vascularity and the cells associated with this vascularity (using techniques such as immunofluorescence and confocal microscopy) only following an ‘event’. It is difficult therefore to say whether the presence of any progenitor cells is the cause of the instability. An alternative explanation for the link between symptomatic carotid artery disease and HPC number and function is that the rise in the latter is an acute phase response to the unstable plaque or ischaemic cerebral tissue found in symptomatic patients. It would be interesting to investigate the effect of removing "symptomatic" elements from plaque on HPC number and function. This could be done by measuring these variables in patients pre and post carotid artery stenting. This would also show

what effect changes to brain perfusion have on HPC number/function and cytokine profile independent of making a large wound and endarterectomising the vessel.

4.6.3 What is an HPC?

A major limitation of this and similar studies is in defining an HPC. The methods used to enumerate these cells use combinations of a variety of markers (including CD31, CD34, VEGFR2, CD62E, CD133, cKit and CD45) and cell culture techniques (early and late CFUs), which makes it impossible to compare studies that attempt to associate HPCs with cardiovascular conditions and outcomes. Though this was evident at the time we began this study what has become apparent over the course of the work is that these techniques clearly measure different cells populations[112, 120, 121, 128, 134]. For example it was once generally accepted that the eCFU assay and $CD133^{+ve}/CD34^{+ve}/VEGFR2^{+ve}$ phenotypic identification by flow cytometry, were two techniques that could be used to identify EPCs. Subsequent studies have shown that these techniques measure a heterogeneous group of primitive haematopoietic progenitor cells (HPCs) that express both endothelial and myeloid lineage markers[120, 128, 197]. We and others[134] have also shown that eCFU analysis and flow cytometric analysis do not correlate suggesting that $CD133^{+ve}/CD34^{+ve}$ cells and those responsible for eCFU formation represent different subsets of HPCs. This raises several important questions:-

- What are these different subtypes?
- What are their relative functions?

- What are their relative contribution to re-endothelialisation and therefore to the development of restenosis?

Although the results of this thesis suggest that cells that form eCFUs and $CD133^{+ve}/CD34^{+ve}$ cells may have role in the attenuation of restenosis, it has clearly failed to answer the question above. Details of the studies that showed the heterogeneity of EPCs and the methods that were used to identify them were published during the course of this study, and we were unable therefore to investigate the importance of these cells (as possible subset of the HPC), given the long-term follow up analysis that we were dependent on in the context of restenosis. For example our study relied on performing the same set of investigations on the entire study population and to change the methodology half way through would have rendered any correlation between HPC analysis and restenosis impossible.

There are a variety of cells that have the capacity to enhance re-endothelialisation either by differentiating into endothelial cells or by secreting factors that promote re-endothelialisation. This raises the question as to which is the optimal cell population to be targeted in any putative local cell enrichment therapy to inhibit restenosis. This will depend both on the particular qualities of the cells and the clinical situation. For example, late CFUs have a very high proliferative potential, but take extended time to appear in culture conditions[121]. Early CFU's do not exhibit the same proliferative potential[121], but as they emerge soon in culture, they

maybe the optimal choice when time is a limiting factor. Conversely late CFUs and mesenchymal stem cells are clearly better suited for ex-vivo expansion given their higher proliferative potential[121]. The characteristics of the different candidate cells and the optimal culture conditions clearly need to be more accurately defined before useful clinical trials can be instigated.

To address this, future studies should concentrate on developing a better understanding of the precise phenotype and function of cells, as well as the local and circulating cytokine milieu that contribute to the maintenance of the endothelium and integrity of the vessel wall following injury. This may be obtained by developing genetically modified mice carrying markers of specific cell phenotypes or knockout/knock-in of specific cytokines postulated to be important in this process. A good example of this is the elucidation of monocyte heterogeneity, with sub-populations of this myeloid cell expressing different phenotypic markers and different functions e.g. the patrolling monocyte[275] .

The colony forming unit is known to be composed of cells of myeloid[121] endothelial[101] and T cell lineages[276] . However, it remains unclear from our study which of these cell types is responsible for the correlation between CFU number and restenosis. Further characterisation (flow cytometric and genomic) should be carried out in an animal model and isolation of individual cell types and their use in interventional studies involving injection into animal models (e.g. fat fed Apoe E

mice) of arterial injury would allow the analysis of their relative importance to the process of re-endothelialisation and the inhibition of restenosis.

4.6.4 HPCs or Endothelial microparticles?

Many studies have used CD34, CD133 and VEGFR2 to define HPCs and have found that HPC number correlates inversely with risk of developing cardiovascular disease and prognosis in established disease [117, 118]. However the use of modern polychromatic flow cytometry (PFC) has called into question not only the true identity of these cells as discussed above, but also whether cells identified using older flow cytometry machines and staining protocols are cells at all. PFC involves the use of up to eight different fluorophores in a single analysis, allowing multiple antigens to be detected and for dead or enucleated cells to be excluded from the analysis. Using a modern PFC protocol a recent study found that true EPCs were extremely rare in the circulation[277]. Reverting back to older staining protocols, the same group then used the cell sorting facility to separate these cells and using an electron microscope found that many of these so called cells were in fact microparticles and extracellular vesicles[277]. These results are difficult to reconcile with earlier papers from the same group[120, 278] that demonstrated that cells identified using flow cytometry could be subsequently grown in culture into endothelial colony forming units[278] and also differentiated into haematopoietic cells of varying lineage[120]. However we must acknowledge that at least some of the 'events' recorded using our flow cytometry protocols may have been microparticles in origin.

Endothelial microparticles (EMP) are vesicles of less than 1μm in diameter that are released from the plasma membranes in response to endothelial activation, injury or apoptosis[279]. Elevated levels of circulating microparticles have been detected in pathological states associated with vascular dysfunction as measured by endothelium-dependent vasodilatation and/or alteration of responsiveness of vascular smooth muscle to vasoconstrictor stimuli [280]. Elevated levels of EMPs, mostly defined as $CD31^+$/Annexin-V^+ or $CD31^+$/CD42- microparticles, have been found in various vascular disorders including coronary, peripheral and cerebrovascular diseases[281-284]. Moreover, EMPs accumulate in atherosclerotic lesions and influence propagation of atherosclerosis [285]. It is possible therefore that EMPs may have an important role to play in regulating endothelial function and therefore intimal hyperplasia.

Future studies should concentrate on using polychromatic flow cytometry to stain for a panel of antigens. This would allow various subsets of HPCs to be identified and correlated with restenosis. In addition EMP markers such as $CD31^+$/Annexin-V^+ or $CD31^+/CD42^-$ should be used to measure microparticles and study their response to arterial injury and restenosis.

4.6.5 The role of SDF-1α

The correlation between SDF-1α levels and the magnitude of post-operative fall in HPCs led us to the hypothesis that higher pre-operative SDF-1α levels lead to increased SDF-1α driven homing of progenitor cells to the site of injury, enhanced

neo-endothelialisation and reduced neointima formation. SDF-1α may therefore have therapeutic potential. The MAGIC cell trial has alerted us to the dangers of systemically injecting a relatively non-specific factor to stimulate progenitor cell mobilisation. This could be avoided by using adenoviruses (probably adeno associated viruses that have less immunogenic and more long lasting activity) or microcapsules to deliver SDF-1α to the site of arterial injury in animal models [286].

4.6.6 Failure to grow OECs and MSCs

Our intention at the start of this study was to measure the number and function of eCFUs, OECs (late CFUs) and MSCs. It was not possible to replicate the methods given in the literature for isolation of OECs[121] or MSCs[190] and as a result we managed only to study eCFU number and function. Both OECs and MSCs have the potential to influence restenosis[130, 131, 183] and future studies should therefore concentrate on refining the research methodology to allow the role of these progenitors in restenosis to be defined.

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Appendix A - Ethics approval and consent form

St Thomas' Hospital Research Ethics Committee

CONSENT FORM FOR PARTICIPATION IN RESEARCH PROJECTS & CLINICAL TRIALS

Title of Project: Tissue and blood samples for the study of aortic and peripheral vascular disease - pathogenesis and circulating markers (occlusive disease)

Principal Investigator: Burnand KG	**Ethics Committee**
Other Investigator/s S Abisi, E Sullivan, S Patel	**Code No**: EC03/098
Enrolling patients:	**Version No:** **Date:**

Outline explanation:

We would like to invite you to take part in a study on the causes of why arteries block or swell and why clots occur.

You are coming into St. Thomas' hospital to have an operation to repair arteries that have furred-up with fat (also called plaques). The plaques that build up in these arteries can become unstable and split, giving rise to symptoms that you may be experiencing. We are interested in how these plaques form, finding a way of detecting which plaques are about to split, as these can be removed by surgery, and why some arteries block while other swell up. We wish to take a sample of your blood sample and to measure levels of substances that are thought to be important in plaque formation and splitting. We also wish to analyse the plaque that is removed as part of your surgery.

We would be grateful if you agreed to take part in our study by consenting to:

1. allowing us to measure the size of your artery using a scanner which involves moving a small probe on the surface of your skin. This is a routine, safe and painless procedure.
2. allowing us to use the tissue that is being removed from your artery as part of your operation and that would normally be thrown away;
3. allowing us to take an extra blood sample. This can be done either when you are having blood taken as part of your routine care or while you are asleep during your operation. The amount of extra blood will be no more than 10 ml (2 teaspoons).
4. **allowing us to take a 30mls blood sample at your first 5 follow-up appointments after your operation.** The tissue and blood may be analysed at St. Thomas' Hospital using a variety of different techniques, some of which may include genetic analysis. This will involve investigating the differences in the composition of genes that we feel may be important in effecting changes in the artery wall, compared with the same genes in people who do not have arterial problems. The samples will be anonymised (coded) and your identity will only be known by the investigators. All information will be kept in the strictest confidence. The sample or the extract from it will be kept for analysis until it is depleted. We wish to stress, however, that by signing this agreement, you will relinquish ownership of any blood or tissue removed as part of your operation and you should not expect to benefit financially from any commercialisation of the research results. You will be referred to a specialist clinic for further advice or treatment if we find any abnormal result in your tests.

We would like to stress that you are under no obligation to take part in this study and that if you do decide to take part, you may stop at any point. Your decision will not affect your treatment.

Thank you for considering our request.

I (name) _____

of (address) _____

I hereby consent to take part in the above investigation, the nature and purpose of which have been explained to me. Any questions I wished to ask have been answered to my satisfaction. I understand that I may withdraw from the investigation at any stage without necessarily giving a reason for doing so and that this will in no way affect the care I receive as a patient

SIGNED (Volunteer) _____ **Date** _____

(Doctor/Investigator) _____ **Date** _____

Appendix B – Updated consent form

15/5/07
Version 4

Patient Identification Number for this trial:

CONSENT FORM

Title of Project: Tissue and blood samples for the study of aortic and peripheral vascular disease - pathogenesis and circulating markers

Name of Researcher: Mr Sanjay Patel

Please initial box

1. I confirm that I have read and understand the information sheet dated 15/5/07 (version 4) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of any of my medical notes and data collected during the study, may be looked at by responsible individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree to take part in the above study. ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

When completed, 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

Appendix C - Data Collection Sheet

Study No

The Role of Vascular Progenitor Cells in Restenosis

Hospital No	Date of Procedure
Date of Sympoms	
DOB	**CEA:**
Sex	Indications
	Stenosis Ipsi: Contr:
BP (Treated?)	Patch or Primary Closure
NIDDM/ IDDM	Local/General
IHD	Shunt
Smoking status	
Cholesterol	
PVD/CVA	
Renal Faliure	

Medication

White Cell Count
C-reactive
Protein
Renal Function
Serum Cholesterol
HDL/LDL

Changes since last visit

Acute Limb ischaemia?
MI/ ACS
Other surgery

Follow Up

Date of next sample Date of next scan

Appendix D – Data Set

Study No	Hospital No	Date of Op	DOB	Age	Sex	Hyper-tension	Diabetes
CEA066	Z602543	27.04.06	22.01.31	75	Male	Yes	no DM
CEA067	1193199D	27.04.06	05.10.29	77	Female	Yes	NIDDM
CEA068	1162248L	27.04.06		71	Male	Yes	NIDDM
CEA077	Z742372	12.07.06	03.10.33	73	Male	Yes	no DM
CEA078	4371698B	20.07.06	30.09.20	86	Female	Yes	NIDDM
CEA079	1813882F	26.07.06	18.04.38	68	Female	No	no DM
CEA080	0605206G	28.07.06		65	Female	Yes	no DM
CEA082	4401101Z	15.08.06	20.03.31	75	Male	Yes	no DM
CEA085	4397599C	30.08.06	11.02.50	56	Male	Yes	no DM
CEA086	0825459C	27.09.06	27.08.37	69	Male	Yes	NIDDM
CEA087	1609846J	28.09.06	19.02.40	66	Male	Yes	no DM
CEA088	1983323D	28.09.06	30.05.44	62	Male	Yes	NIDDM
CEA091	4396210X	02.11.06	12.08.37	69	Male	No	NIDDM
CEA092	4428833C	01.11.06	14.01.28	78	Male	Yes	no DM
CEA093	1405865T	02.11.06	05.08.21	85	Female	Yes	no DM
CEA094	1142857Z	02.11.06	05.11.34	72	Male	Yes	no DM
CEA095	1335568X	07.11.06	06.02.36	70	Female	Yes	no DM
CEA096	4410677B	08.11.06	24.06.45	61	Female	Yes	no DM
CEA097	4429010P	09.11.06	03.01.46	60	Male	Yes	no DM
CEA100	Z758272	30.11.06	05.04.25	81	Male	No	NIDDM
CEA101	4327740G	30.11.06	11.09.37	69	Male	Yes	NIDDM
CEA102	4429556F	01.12.06	14.10.37	69	Male	Yes	no DM
CEA105	4471146W	24.01.07	16.07.26	80	Male	Yes	no DM
CEA106	0366578S	21.02.07	16.09.25	82	Male	No	no DM
CEA107	Z236279	22.02.07	14.05.37	69	Female	Yes	NIDDM
CEA108	1609825B	15.03.07	18.08.33	73	Female	Yes	no DM
CEA109	2211068L	24.04.07	05.10.52	64	Male	No	no DM
CEA110	1275109G		28.11.35	71	Male	No	no DM
CEA111	4487659W		29.04.30	76	Male	Yes	no DM
CEA112	4256621D	04.05.07	20.11.41	65	Female	Yes	no DM
CEA113	4490242E		22.06.45	61	Male	Yes	no DM
CEA114	Z010582	16.05.07	16.03.23	84	Male	Yes	no DM
CEA115	1288000U	24.05.07	13.04.40	67	Female	No	no DM
CEA116	4514641W	24.05.07	13.10.29	77	Female	Yes	no DM
CEA117	4501618R	24.05.07	16.02.36	71	Female	Yes	NIDDM
CEA119	2124705L	06.06.07	17.07.39	68	Male	Yes	no DM

Study No	Hospital No	Date of Op	DOB	Age	Sex	Hyper-tension	Diabetes
CEA120	1433802F	13.06.07	08.09.40	67	Female	Yes	NIDDM
CEA126	2239612G	30.07.07	22.12.35	72	Female	No	no DM
CEA127	4541559G	30.07.07	29.02.40	67	Male	Yes	no DM
CEA128	1372408J	09.08.07	16.01.24	83	Female	Yes	no DM
CEA129	4546778D	09.08.07	10.06.46	61	Female	Yes	NIDDM
CEA130	4548033S	15.08.07	21.09.44	67	Male	Yes	NIDDM
CEA131	4555650K	16.08.07	14.03.34	73	Male	Yes	no DM
CEA132	Z676521	18.08.07	15.05.19	88	Female	Yes	no DM
CEA133	4418582X	29.08.07	19.03.32	75	Male	No	no DM
CEA134	2248991W	18.09.07	25.03.47	60	Male	Yes	no DM
CEA135	2254153G	25.10.07	26.04.54	53	Male	Yes	no DM
CEA136	4579477G	25.10.07	20.08.41	66	Male	No	no DM
CEA137	4595987W	20.11.07	25.02.28	79	Male	Yes	no DM
CEA138	4498914E	21.11.07	03.10.52	55	Male	No	no DM
CEA139	1466038E	02.12.07	09.01.41	66	Male	Yes	NIDDM
CEA140	Z376513	13.12.07	12.03.29	78	Male	No	no DM
CEA141	4602162W	20.12.07	19.03.43	64	Male	Yes	no DM
CEA142	22587097	20.12.07	06.12.44	63	Male	No	no DM
CEA143	Z448913	15.02.08	03.03.32	75	Male	Yes	NIDDM
CEA144	0862345T	15.02.08	20.06.43	64	Male	No	no DM
CEA145	Z612772	12.02.08	04.03.30	77	Male	Yes	no DM
CEA146	Z576760	20.02.08	10.05.20	87	Male	Yes	no DM
CEA147	0290543B	21.02.08	14.12.23	84	Male	Yes	no DM
CEA148	0308220X	21.02.08	22.05.45	62	Male	Yes	no DM
CEA149	4612392X	13.03.08	23.01.24	83	Male	No	no DM
CEA150	4335952Q	13.03.08	01.08.48	59	Male	Yes	no DM

Study No	IHD	Smoker	High Cholesterol	PVD	Renal Failure
CEA066	No IHD	Ex Smoker	Yes	no	No
CEA067	No IHD	Ex Smoker	Yes	no	No
CEA068	Angina	Ex Smoker	Yes	no	No
CEA077	No IHD	Ex Smoker	Yes	Yes	No
CEA078	No IHD	Ex Smoker	Yes	no	No
CEA079	No IHD	Non Smoker	Yes	no	No
CEA080	No IHD	Current Smoker	Yes	no	No
CEA082	No IHD	Ex Smoker	Yes	no	No
CEA085	No IHD	Ex Smoker	Yes	no	No
CEA086	MI	Current Smoker	Yes	no	No
CEA087	MI	Current Smoker	Yes	no	No
CEA088	No IHD	Ex Smoker	Yes	Yes	Yes
CEA091	MI	Ex Smoker	Yes	no	No
CEA092	MI	Ex Smoker	Yes	no	No
CEA093	No IHD	Non Smoker	Yes	no	Yes
CEA094	No IHD	Non Smoker	Yes	no	No
CEA095	MI	Current Smoker	Yes	no	No
CEA096	No IHD	Current Smoker	Yes	Yes	No
CEA097	No IHD	Current Smoker	Yes	Yes	No
CEA100	MI	Non Smoker	No	no	No
CEA101	Angina	Non Smoker	Yes	no	No
CEA102	No IHD	Ex Smoker	Yes	no	No
CEA105	No IHD	Ex Smoker	No	Yes	Yes
CEA106	MI	Non Smoker	Yes	no	No
CEA107	MI	Non Smoker	Yes	no	No
CEA108	MI	Non Smoker	Yes	no	No
CEA109	No IHD	Ex Smoker	No	no	No
CEA110	MI	Current Smoker	Yes	no	No
CEA111	No IHD	Ex Smoker	Yes	no	No
CEA112	No IHD	Non Smoker	Yes	no	No
CEA113	No IHD	Ex Smoker	Yes	no	No
CEA114	Angina	Ex Smoker	Yes	no	No
CEA115	MI	Non Smoker	No	no	No
CEA116	MI	Ex Smoker	Yes	Yes	No
CEA117	No IHD	Non Smoker	Yes	no	No
CEA119	No IHD	Current Smoker	No	no	No
CEA120	No IHD	Non Smoker	Yes	no	No

Study No	IHD	Smoker	High Cholesterol	PVD	Renal Failure
CEA126	No IHD	Non Smoker	Yes	no	No
CEA127	No IHD	Ex Smoker	Yes	no	No
CEA128	No IHD	Ex Smoker	Yes	no	No
CEA129	Angina	Ex Smoker	Yes	Yes	No
CEA130	MI	Ex Smoker	Yes	no	No
CEA131	No IHD	Ex Smoker	Yes	no	No
CEA132	No IHD	Non Smoker	Yes	no	No
CEA133	No IHD	Ex Smoker	Yes	Yes	No
CEA134	No IHD	Non Smoker	Yes	no	No
CEA135	No IHD	Current Smoker	Yes	no	No
CEA136	Angina	Ex Smoker	Yes	no	No
CEA137	No IHD	Ex Smoker	Yes	no	No
CEA138	MI	Ex Smoker	Yes	no	No
CEA139	No IHD	Current Smoker	Yes	no	No
CEA140	MI	Current Smoker	Yes	Yes	No
CEA141	Angina	Current Smoker	Yes	no	No
CEA142	No IHD	Non Smoker	Yes	no	No
CEA143	MI	Ex Smoker	Yes	Yes	No
CEA144	No IHD	Current Smoker	Yes	no	No
CEA145	Angina	Ex Smoker	Yes	Yes	No
CEA146	No IHD	Ex Smoker	Yes	no	Yes
CEA147	MI	Ex Smoker	Yes	no	No
CEA148	MI	Non Smoker	Yes	no	No
CEA149	Angina	Ex Smoker	Yes	Yes	No
CEA150	MI	Ex Smoker	Yes	no	No

Study No	Aspirin	Warfarin	Clopid-ogrel	Statin	ACEi	ATIIAnt	Digoxin	B - Blocker
CEA066	Yes	No	No	Yes	no	No	No	No
CEA067	Yes	No	No	Yes	no	Yes	No	No
CEA068	Yes	Yes	No	Yes	Yes	No	No	Yes
CEA077	Yes	No	No	Yes	no	No	No	No
CEA078	Yes	No	No	Yes	no	Yes	No	Yes
CEA079	Yes	No	Yes	Yes	Yes	No	Yes	No
CEA080	Yes	No	No	Yes	no	Yes	No	No
CEA082	Yes	No	No	Yes	no	Yes	No	No
CEA085	Yes	No	Yes	Yes	Yes	No	No	Yes
CEA086	Yes	No	No	Yes	Yes	No	No	No
CEA087	Yes	No	No	Yes	no	No	No	Yes
CEA088	Yes	No	No	Yes	no	No	No	Yes
CEA091	Yes	No	No	Yes	no	No	No	Yes
CEA092	Yes	No	No	Yes	no	No	No	Yes
CEA093	No	Yes	No	Yes	Yes	No	No	Yes
CEA094	Yes	No	No	Yes	no	No	No	No
CEA095	No	Yes	No	Yes	Yes	No	No	Yes
CEA096	No	No	Yes	Yes	Yes	No	No	No
CEA097	No	Yes	No	Yes	Yes	No	No	No
CEA100	Yes	No	No	Yes	no	No	No	Yes
CEA101	Yes	Yes	No	Yes	no	No	No	Yes
CEA102	Yes	No	No	Yes	no	No	No	No
CEA105	No	No	Yes	No	Yes	No	No	No
CEA106	Yes	No	No	Yes	no	No	No	Yes
CEA107	No	Yes	No	Yes	Yes	No	No	Yes
CEA108	No	Yes	No	Yes	Yes	No	No	No
CEA109	Yes	No	No	No	no	No	No	No
CEA110	Yes	No	No	Yes	no	No	No	Yes
CEA111	No	Yes	No	Yes	Yes	No	No	No
CEA112	Yes	No	No	Yes	Yes	No	No	Yes
CEA113	Yes	No	No	Yes	Yes	No	No	Yes
CEA114	Yes	No	Yes	Yes	no	No	No	No
CEA115	No	No	Yes	No	no	No	No	Yes
CEA116	Yes	Yes	No	Yes	no	No	No	Yes
CEA117	No	No	Yes	Yes	Yes	Yes	No	No
CEA119	Yes	No	No	No	Yes	No	No	No
CEA120	No	No	No	Yes	no	Yes	No	No

Study No	Aspirin	Warfarin	Clopid-ogrel	Statin	ACEi	ATIIAnt	Digoxin	B - Blocker
CEA126	Yes	No	No	Yes	no	No	No	No
CEA127	Yes	No	No	Yes	Yes	No	No	No
CEA128	Yes	No	Yes	Yes	Yes	No	No	No
CEA129	No	No	Yes	Yes	Yes	No	No	Yes
CEA130	Yes	Yes	No	Yes	no	Yes	No	Yes
CEA131	No	Yes	No	Yes	Yes	No	No	No
CEA132	Yes	No	No	Yes	no	No	No	No
CEA133	No	Yes	No	Yes	no	No	No	No
CEA134	Yes	No	No	Yes	no	No	No	No
CEA135	Yes	No	No	Yes	no	No	No	No
CEA136	Yes	No	No	Yes	no	No	No	No
CEA137	No	Yes	No	Yes	no	No	No	Yes
CEA138	Yes	No	No	Yes	no	No	No	No
CEA139	Yes	No	No	Yes	Yes	No	No	No
CEA140	Yes	No	No	Yes	Yes	No	No	No
CEA141	Yes	No	No	Yes	no	No	No	Yes
CEA142	Yes	No	No	Yes	no	No	No	No
CEA143	Yes	No	No	Yes	Yes	No	No	Yes
CEA144	Yes	No	No	No	no	No	No	No
CEA145	No	Yes	No	Yes	Yes	No	No	No
CEA146	Yes	No	No	Yes	Yes	No	No	No
CEA147	Yes	No	No	Yes	Yes	No	No	Yes
CEA148	Yes	Yes	Yes	Yes	Yes	No	No	Yes
CEA149	Yes	No	No	Yes	no	No	No	Yes
CEA150	No	Yes	No	Yes	Yes	No	No	Yes

Study No	Diuretic	Ca Channel	Indication	Ipsilateral Stenosis	Contralateral Stenosis	Time to Surgery (weeks)
CEA066	No	no	CVA	50	100	7
CEA067	yes	Yes	Asymptomatic	80	60	0
CEA068	No	no	TIA	79	40	7
CEA077	yes	no	CVA	79	49	5
CEA078	yes	no	CVA	50	100	10
CEA079	No	no	TIA	70	30	7
CEA080	No	no	CVA	80		7
CEA082	No	no	CVA	90	49	7
CEA085	No	Yes	TIA	70	40	8
CEA086	No	Yes	CVA	70	20	7
CEA087	yes	Yes	Asymptomatic	75	16	0
CEA088	yes	Yes	CVA	70	60	7
CEA091	No	no	TIA	90	50	24
CEA092	yes	Yes	TIA	90	100	24
CEA093	No	no	TIA	79	79	7
CEA094	yes	Yes	Asymptomatic	70	50	0
CEA095	No	no	Amaurosis Fugax	80	75	16
CEA096	No	no	Asymptomatic	90	100	9
CEA097	No	Yes	Asymptomatic	90	50	0
CEA100	No	no	Amaurosis Fugax	70	40	6
CEA101	No	Yes	Amaurosis Fugax	70	70	60
CEA102	yes	Yes	Asymptomatic	70	40	0
CEA105	yes	no	Amaurosis Fugax	99	50	41
CEA106	No	no	CVA	70	30	14
CEA107	No	no	TIA	75	60	7
CEA108	yes	Yes	TIA	90	100	8
CEA109	No	no	CVA	70		1
CEA110	No	no	TIA	85	15	8
CEA111	yes	no	TIA	65	20	28
CEA112	yes	no	TIA	75		2
CEA113	No	no	CVA	80	40	8
CEA114	No	Yes	Asymptomatic	90	60	0
CEA115	No	no	TIA	95	70	1

Study No	Diuretic	Ca Channel	Indication	Ipsilateral Stenosis	Contralateral Stenosis	Time to Surgery (weeks)
CEA116	No	no	CVA	75	50	3
CEA117	No	Yes	CVA	80	50	27
CEA119	No	Yes	TIA	80	40	12
CEA120	yes	Yes	TIA	80	30	4
CEA126	yes	no	CVA	90	20	2
CEA127	yes	Yes	CVA	80	50	1
CEA128	No	Yes	TIA	70		12
CEA129	yes	Yes	Asymptomatic	90	75	0
CEA130	yes	Yes	Asymptomatic	90	80	0
CEA131	No	no	TIA	70	20	8
CEA132	No	Yes	CVA	70	30	2
CEA133	No	no	TIA	85	20	1
CEA134	No	no	CVA	90		1
CEA135	No	no	TIA	95	65	2
CEA136	No	no	Asymptomatic	90	80	0
CEA137	No	no	CVA	90	80	2
CEA138	No	no	Asymptomatic	49	100	0
CEA139	No	no	Asymptomatic	90	80	0
CEA140	No	no	TIA	90	90	4
CEA141	No	no	Asymptomatic	80	70	0
CEA142	No	no	CVA	80		5
CEA143	No	no	Asymptomatic	70	30	0
CEA144	No	no	CVA	70	50	8
CEA145	yes	Yes	Asymptomatic	79	40	0
CEA146	yes	Yes	CVA	85	75	3
CEA147	No	no	Asymptomatic	70	50	0
CEA148	yes	no	CVA	70		7
CEA149	yes	Yes	Asymptomatic	99	80	0
CEA150	No	Yes	Asymptomatic	75	60	0

Study No	Plaque Morphology	1yr Oxford Risk	5yr Oxford Risk	Side	Patch	LA/GA	Shunt
CEA066	Ulcerated/Irregular	11.4	28.3	Right	No Patch	GA	Shunt
CEA067	Ulcerated/Irregular			Left	Patch	GA	No Shunt
CEA068	Ulcerated/Irregular	15.9	38	Right	No Patch	GA	No Shunt
CEA077	Ulcerated/Irregular	8.3	21.2	Left	Patch	GA	Shunt
CEA078	Smooth	6.5	16.8	Left	Patch	GA	Shunt
CEA079	Ulcerated/Irregular	5.6	14.9	Left	Patch	GA	Shunt
CEA080	Ulcerated/Irregular			Left	No Patch	GA	No Shunt
CEA082	Ulcerated/Irregular	15.4	36.9	Right	No Patch	GA	No Shunt
CEA085	Ulcerated/Irregular	7	18.2	Left	Patch	GA	No Shunt
CEA086	Ulcerated/Irregular	20	48.5	Left	Patch	GA	Shunt
CEA087	Ulcerated/Irregular			Right	Patch	GA	No Shunt
CEA088	Ulcerated/Irregular	15.4	36.9	Right	No Patch	GA	Shunt
CEA091	Ulcerated/Irregular	9	22.8	Left	Patch	GA	Shunt
CEA092	Ulcerated/Irregular	9.1	30	Left	No Patch	GA	Shunt
CEA093	Smooth	5	12.8	Right	No Patch	LA	No Shunt
CEA094	Smooth			Right	No Patch	GA	Shunt
CEA095	Smooth	5	12.1	Right	Patch	GA	No Shunt
CEA096	Ulcerated/Irregular			Left	Patch	GA	Shunt
CEA097	Ulcerated/Irregular			Left	No Patch	GA	No Shunt
CEA100	Ulcerated/Irregular	16.3	38.7	Right	No Patch	GA	No Shunt
CEA101	Ulcerated/Irregular			Right	Patch	GA	No Shunt
CEA102	Ulcerated/Irregular			Right	No Patch	GA	No Shunt
CEA105	Ulcerated/Irregular			Right	Patch	GA	Shunt
CEA106	Ulcerated/Irregular	14	34	Right	Patch	GA	Shunt
CEA107	Smooth	8	20.5	Left	Patch	GA	Shunt
CEA108	Smooth	7.9	20	Right	Patch	GA	No Shunt
CEA109	Ulcerated/Irregular	10.5	26.4	Left	Patch	LA	No Shunt
CEA110	Ulcerated/Irregular	12.9	31.7	Left	Patch	GA	Shunt
CEA111	Ulcerated/Irregular	5	11.1	Left	Patch	LA	No Shunt
CEA112	Ulcerated/Irregular	9	22.8	Left	No Patch	GA	No Shunt
CEA113	Ulcerated/Irregular	11	27.6	Right	Patch	LA	No Shunt
CEA114	Smooth			Right	Patch	GA	Shunt
CEA115	Ulcerated/Irregular	10.6	26.5	Left	Patch	GA	No Shunt
CEA116	Ulcerated/Irregular	20	49.2	Left	Patch	LA	No Shunt
CEA117	Smooth	5	10	Right	Patch	GA	No Shunt
CEA119	Ulcerated/Irregular	7.9	20.3	Left	Patch	GA	Shunt

Study No	Plaque Morphology	1yr Oxford Risk	5yr Oxford Risk	Side	Patch	LA/GA	Shunt
CEA120	Ulcerated/Irregular	12.1	30	Left	Patch	GA	Shunt
CEA126	Smooth	6.4	16.7	Left	Patch	LA	No Shunt
CEA127	Smooth	7.9	20.4	Left	Patch	GA	No Shunt
CEA128	Ulcerated/Irregular	6.7	17.3	Left	Patch	GA	Shunt
CEA129	Smooth			Right	Patch	GA	No Shunt
CEA130	Ulcerated/Irregular			Right	Patch	GA	Shunt
CEA131	Ulcerated/Irregular	8.4	21.4	Left	No Patch	GA	No Shunt
CEA132	Ulcerated/Irregular	13.4	32.8	Left	No Patch	GA	No Shunt
CEA133	Ulcerated/Irregular	13.5	33	Left	No Patch	LA	No Shunt
CEA134	Smooth	8.6	22	Left	No Patch	GA	No Shunt
CEA135	Ulcerated/Irregular	12.7	31.3	Right	Patch	GA	No Shunt
CEA136	Ulcerated/Irregular			Left	No Patch	GA	No Shunt
CEA137	Ulcerated/Irregular	19.4	44.8	Left	No Patch	LA	No Shunt
CEA138	Ulcerated/Irregular			Left	Patch	GA	Shunt
CEA139	Smooth			Right	Patch	GA	Shunt
CEA140	Ulcerated/Irregular	19.5	44.9	Left	Patch	LA	No Shunt
CEA141	Smooth			Left	No Patch	LA	No Shunt
CEA142	Ulcerated/Irregular	10.3	26	Right	Patch	GA	No Shunt
CEA143	Smooth			Left	Patch	GA	Shunt
CEA144	Ulcerated/Irregular	7.8	20.2	Left	Patch	GA	Shunt
CEA145	Ulcerated/Irregular			Right	Patch	GA	No Shunt
CEA146	Ulcerated/Irregular	18.8	43.6	Left	Patch	GA	No Shunt
CEA147	Smooth			Left	No Patch	GA	No Shunt
CEA148	Ulcerated/Irregular	15.2	36.5	Left	No Patch	GA	No Shunt
CEA149	Smooth			Right	No Patch	GA	No Shunt
CEA150	Smooth			Left	No Patch	GA	No Shunt

Study No	WCC	Neutro-phils	Lympho-cytes	Mono-cytes	CRP	Urea	Creatinine	Cholesterol
CEA066	6.2	3.8	1.6	0.6	8	7.7	99	3.5
CEA067	10.3	7.2	2.4	0.5	6	7.2	97	3.8
CEA068	5	2.6	2	0.5	5	14	102	
CEA077	6.2	3.9	1.7	0.5	1	4.5	94	3.6
CEA078	11.6	9.3	1.5	0.7	1	11.1	109	3.7
CEA079	13.5	11.3	1.8	0.4	10	4.3	67	
CEA080	8.4	5.8	1.6	0.9	1	6.2	86	
CEA082	4	2.4	1	0.04	1	6.2	90	4.7
CEA085	7.5	5.3	1.7	0.4	1	5.4	91	2.6
CEA086	13.3	12.4	0.5	0.5	1	5.1	93	3.4
CEA087	8.7	5	2.7	0.7	12	5.5	141	2.4
CEA088	10.8	7	1.9	1.1	7	7.6	135	3.4
CEA091	8.6	5.8	1.9	0.6	1	6.3	122	4.8
CEA092	5.5	3.8	1.2	0.4	79	6.1	107	
CEA093	7.7	5.9	0.7	0.6	56	13.9	510	3.4
CEA094	5.3	3.3	1.4	0.5	1	6.1	65	3.1
CEA095	9.6	8.4	0.9	0.2	17	9.6	72	
CEA096	8.5	4.7	3.1	0.7	1	5.3	69	
CEA097	12.3	11.4	0.2	0.5	1	5.4	83	4.5
CEA100	9.5	5.5	2.9	0.8	1	6.2	96	3.3
CEA101	5.5	2.8	1.7	0.9	1	3.9	66	3
CEA102	12.1	7.9	2.8	0.7	1	4.6	81	
CEA105	8.6	6.1	1.7	0.7	1	9.5	128	
CEA106	8.5	4.9	2.6	0.7	1	6.7	94	3.8
CEA107	8.8	6.9	1.2	0.5	6	7	73	4.3
CEA108	8.1	4.6	2.3	0.9	1	6	83	4.2
CEA109	5.9	3.5	1.5	0.6	10	6.3	51	4.9
CEA110	6.7	3.6	2.1	0.7	5	6.3	74	
CEA111	6.2	5	0.7	0.4	9	9.92	180	
CEA112	9.7	5.9	2.6	0.9	13	7.1	90	
CEA113	10	6.5	2.5	0.7	1	7.1	108	
CEA114	7	4.8	1.1	0.7	1	5.3	70	
CEA115	5.9	2.7	2.3	0.6	12	5.8	86	5.9
CEA116	7.3	5.2	1.5	0.5	1	6.5	110	4.1
CEA117	9.1	5.3	3.1	0.5	1	7.3	88	
CEA119	7.3	4.1	2.3	0.7	1	3.2	66	4.3
CEA120	5.1	2.2	2	0.5	1	4.9	73	4.2

Study No	WCC	Neutro-phils	Lympho-cytes	Mono-cytes	CRP	Urea	Creatinine	Cholesterol
CEA126	10.1	6.7	2.5	0.7	1	5.4	98	5.9
CEA127	6.4	4.3	1	0.8	37	8.2	170	4
CEA128	11.9	10.5	0.8	0.6	1	3.2	68	
CEA129	5.8	3	2	0.6	1	8.3	76	
CEA130	7.4	4.7	2	0.6	1	8.9	109	3.9
CEA131	9.6	5.8	2.6	1	1	5.5	105	3.3
CEA132	7.6	5.5	1.4	0.6	1	5.4	62	4.3
CEA133	7.8	5.5	1.3	0.9	1	3.7	77	4.4
CEA134	6.8	3.3	2.8	0.7	1	5.6	108	7.6
CEA135	6.7	3.7	2	0.8	1	6.1	111	3.4
CEA136	9.1	6.4	1.9	0.6	22	5.8	80	
CEA137	6.9	4.8	1.2	0.6	1	5.3	108	
CEA138	8.4	5.5	2.1	0.7	1	3.7	96	4.5
CEA139	11.8	6.6	3.5	0.8	1	7.7	105	
CEA140	8.1	5.4	1.5	1	77	5.5	83	
CEA141	8.3	4.8	2.5	0.7	1	6	74	
CEA142	7.2	4.7	1.7	0.6	1	6.2	77	
CEA143	5.8	2.7	2.6	0.4	1	6.3	97	2.8
CEA144	10.2	7.1	1.7	1	8	7.5	95	5.9
CEA145	9.9	6.8	1.9	1	1	8.4	112	
CEA146	6.3	5.2	0.8	0.3	1	15.6	159	
CEA147	12.8	9.5	2.4	0.8	1	7.1	72	1.7
CEA148	6.3	4	1.6	0.5	1	7.2	81	
CEA149	11.5	7.8	2.3	0.9	7	12.6	130	4.5
CEA150	12.6	8.1	1.3	0.7	1	5.4	81	3.7

Study No	CD133 pre	CD34 pre	Double pre	WCC post	Neutrophil post	Lymphocyte post	Monocytes post
CEA066	195	220	162	7.5	5.1	1.5	0.8
CEA067	527	594	508				
CEA068	177	221	159	4.5	2.7	1.4	0.4
CEA077	137	113	80	9.7	7.2	1.6	0.6
CEA078	63	103	42				
CEA079	80	72	70				
CEA080	199	241	193	15.2	12.9	1.2	1.2
CEA082	191	199	175				
CEA085	168	197	148				
CEA086	121	137	110	10.3	7.1	2.1	1.1
CEA087	64	68	55	8.4	5.7	1.9	0.5
CEA088	95	97	76	12	8.5	1.7	1
CEA091	172	187	141	10.1	7.4	1.8	0.6
CEA092	211	232	189	8.9	6.4	1.6	0.8
CEA093	122	193	131	6.3	3.2	1.1	0.7
CEA094	109	147	102	9.2	8	0.8	0.4
CEA095	124	81	74	16.6	14.3	1.3	1
CEA096	60	80	55	8.4	5.5	2.3	0.5
CEA097	255	198	161	11.6	10.6	0.5	0.5
CEA100	44	77	46	11.1	8.3	1.8	0.9
CEA101	92	139	71	6.6	4.2	1.5	0.8
CEA102	434	542	429	12.3	8.1	2.6	0.9
CEA105	238	334	229	8.6	6.1	1.7	0.7
CEA106	487	494	457	18.7	16.8	1.1	0.6
CEA107	51	72	51	11.3	10.1	0.9	0.3
CEA108	194	252	181	8.2	5.4	1.6	0.8
CEA109	212	285	212	7.5	4.8	2.1	0.5
CEA110	81	191	65	11.9	10	1.4	0.6
CEA111	202	251	199	7.9	6.8	0.7	0.4
CEA112	133	155	128	7.5	5.6	1.4	0.5
CEA113	79	112	82				
CEA114	105	180	111	15.9	14.6	0.6	0.6
CEA115	164	161	138	9	6.2	1.7	0.8
CEA116	188	186	122	5	3.1	1.2	0.6
CEA117	128	72	53	10.6	7.8	2	0.5
CEA119	101	130	57	12	9.7	1.6	0.7
CEA120	161	111	32	11.1	8.3	1.9	0.8

Study No	CD133 pre	CD34 pre	Double pre	WCC post	Neutrophil post	Lymphocyte post	Monocytes post
CEA126	131	99	87	7.9	5.4	1.7	0.6
CEA127	426	205	158	7.5	5.6	1	0.8
CEA128	180	182	164	7.3	5.4	1.2	0.4
CEA129	175	170	146	8.8	5.5	2.3	0.8
CEA130	102	98	53	12.1	9.8	1.3	1
CEA131	80	33	33	9.9	6.5	2	1
CEA132	45	58	34	8.3	6.5	1	0.7
CEA133	45	86	50	6.7	4.8	1	0.8
CEA134	243	343	240	7.5	5.6	1.2	0.5
CEA135	127	173	130	16.1	12.4	1.8	2.1
CEA136	180	156	151	12.9	11.9	0.9	0.1
CEA137	123	124	98	7.1	5.1	1.1	0.8
CEA138	94	139	121	18.2	16.4	1.1	0.7
CEA139	105	125	105	17.6	16	0.9	0.7
CEA140	106	133	102				
CEA141	184	199	163	9.4	7.7	1	0.7
CEA142	80	94	81	13.5	10.9	1.6	0.9
CEA143	64	79	68	7.1	4.7	1.8	0.5
CEA144	64	79	68	12.4	8.7	2.1	1.2
CEA145	303	356	251	10.9	8.4	1.3	1.2
CEA146	35	125	65				
CEA147	46	80	63	15.1	13.3	1.5	0.3
CEA148	122	144	100	7.8	7	0.6	0.1
CEA149	223	313	223	12.6	10.2	1.3	0.9
CEA150	135	215	142				

Study No	CD133 post	CD34 post	Double post	CD133 6weeks	CD34 6weeks	Double 6weeks
CEA066	167	218	148			
CEA067	326	407	293			
CEA068	126	159	114			
CEA077	86	109	88	77	111	71
CEA078	92	123	85			
CEA079	211	278	224	96	116	195
CEA080	138	215	133	106	108	85
CEA082	178	185	142			
CEA085	109	169	86	156	164	133
CEA086	107	132	69	137	174	133
CEA087	27	31	23	38	44	26
CEA088	63	82	55	107	102	76
CEA091	96	116	79	118	131	100
CEA092	178	203	152			
CEA093	159	190	150	170	228	161
CEA094	83	102	59	115	142	104
CEA095	48	43	32	82	104	73
CEA096	25	39	19	50	82	52
CEA097	149	138	109			
CEA100	26	54	37	51	75	47
CEA101	60	94	55	89	90	79
CEA102	218	288	204	364	520	363
CEA105	173	273	177			
CEA106	259	312	252	455	554	436
CEA107	57	69	54	79	77	61
CEA108	194	210	180			
CEA109	179	278	181			
CEA110	34	45	25	64	77	50
CEA111	188	248	189	233	287	206
CEA112	148	174	138			
CEA113	96	131	97			
CEA114	58	129	61	54	122	56
CEA115	148	140	123			
CEA116	131	129	86			
CEA117	174	109	72			
CEA119	85	112	54	76	87	50
CEA120	72	74	8	37	67	24

Study No	CD133 post	CD34 post	Double post	CD133 6weeks	CD34 6weeks	Double 6weeks
CEA126	135	86	78			
CEA127	85	85	75	210	172	188
CEA128	196	164	132	100	87	65
CEA129	302	59	41			
CEA130	53	52	34	116	114	78
CEA131	120	10	13	80	29	27
CEA132	56	87	49	34	50	27
CEA133	32	48	33			
CEA134	208	261	190	255	292	245
CEA135	38	68	45			
CEA136	96	116	76			
CEA137	91	105	74			
CEA138	84	86	82	15	61	40
CEA139	143	195	157	22	85	49
CEA140	88	134	90	55	96	62
CEA141	126	149	117	36	78	65
CEA142	75	69	55	80	87	73
CEA143	70	64	54	184	225	161
CEA144	70	64	54			
CEA145	237	413	192			
CEA146						
CEA147	47	54	39			
CEA148	79	88	61			
CEA149	147	211	155			
CEA150	99	122	100			

Study No	CFU pre	CFU post	CFU 6 week	Bgal Pre	Bgal post	Bgal 6 weeek	Migration pre	Migration post	Migration 6weeks
CEA066									
CEA067									
CEA068									
CEA077	16	10	17						
CEA078	14	11							
CEA079	16	16	14						
CEA080	15	9	14						
CEA082	12	11							
CEA085	14	12	16	18.2	14.2	26.7			
CEA086	13	11	14	29.7	33.6				
CEA087	17			23.4	19.4				
CEA088	18	16	16	23.5	21.9	24.2			
CEA091	16	10	14	22.6	29.7	31.3			
CEA092	15	16		27.1	22.5				
CEA093	16	8	18	34.1	33.1	29.8			
CEA094	18	16	16	28.3	26.8	30.2	1.04	1.08	1.12
CEA095	14	10	12	28.2	23.3	26.7	1.26	1.29	1.22
CEA096	17	13	16	23.6	27.5	28.1	1.1	1.12	1.14
CEA097	12	12		19.9	16.6		1.14	1.11	
CEA100	17	10	16	32.4	28.5	26.2	1.17	1.15	1.19
CEA101	13	11	13	24.4	26.9	22.8	1.15	1.19	1.07
CEA102	16	12	15	22.7		27.7	1.24		1.28
CEA105	14	10		27.9	29.3		1.19	1.17	
CEA106	16	15	16	29.5	24.4		1.16	1.16	
CEA107	16	13	18	26.4	26.1		1.23	1.16	
CEA108	12	12		28.3	22.5		1.05	1.11	
CEA109	21	11		22.9	31.4		1.29	1.25	
CEA110	11	7	12	29.8	33	35.2	1.32	1.33	1.28
CEA111	21	18	22	26.1	27	24.2	1.17	1.19	1.16
CEA112	22	24		23.4	25.6		1.09	1.11	
CEA113	19	19		20.4	22.1		1.14	1.1	
CEA114	10	10	10	34.1		31	1.13		1.15
CEA115	23	21		26.1			1.17		
CEA116	16	15		33.2	37.7		1.21	1.26	
CEA117	18	11		27.5	25.5		1.33	1.28	
CEA119	19	17	18	24.9	21	26.1	1.19	1.24	1.2
CEA120	15	10	14	24.3	22.5	26.8	1.24	1.22	1.18

Study No	CFU pre	CFU post	CFU 6 weeks	Bgal Pre	Bgal post	Bgal 6 weeeks	Migration pre	Migration post	Migration 6weeks
CEA126				31.2	33.9		1.08	1.12	
CEA127	16	14	19	28.1	28.4	24.3	1.4	1.33	1.41
CEA128	18	15	19	27.4	25.3	22.3	1.35	1.31	1.3
CEA129	11	12		25.1	21.4		1.08	1.14	
CEA130	14	11	13	26.7			1.1		
CEA131	18	12	18	25.3	29	27.4	1.56	1.51	1.46
CEA132	24	18	22	32.1	28.1	26.8	1.14	1.08	1.16
CEA133	17	11		31.4	35.7		1.29	1.27	
CEA134	18	16	20	17.1		23.3	1.26		1.32
CEA135	24	20		18.9			1.21		
CEA136	12	12		24.7	22.6		1.14	1.14	
CEA137	21	19		31.7	26		1.34	1.28	
CEA138	14	11	16	22.8	25.3	28.4	1.18	1.17	1.15
CEA139	12	11	13	26.4	21.8	28.5	1.17	1.34	1.19
CEA140	18	15	16	24.7	24	22	1.32	1.33	1.34
CEA141	13	11	12	24.4	27.3	31.2	1.14	1.11	1.09
CEA142	22	18	24	22.2	15.1	19.3	1.19	1.22	1.2
CEA143	14	12	13	28.1	24.4	26.9	1.12	1.12	1.17
CEA144	18	15		26.7	21.9		1.19	1.17	
CEA145	15	12		29.9			1.15		
CEA146	16	15		31.1	27.2		1.25	1.23	
CEA147	11	10		33.1	37		1.07	1.12	
CEA148	19	15		24.3	22.4		1.28	1.26	
CEA149	10	8		34.7			1.09		
CEA150	11	9		23.1	26.1		1.14	1.12	

Study No	GMCSF pre	GMCSF post	GMCSF 6weeks	PlGF pre	PlGF post	PlGF 6weeks	SDF pre	SDF post	SDF 6weeks
CEA066									
CEA067									
CEA068									
CEA077									
CEA078									
CEA079									
CEA080									
CEA082									
CEA085									
CEA086									
CEA087									
CEA088									
CEA091									
CEA092									
CEA093									
CEA094	133	393.8	372	15.8	23.2	33.2	1547.6	644.4	702.2
CEA095	74.2	286.8	730.4	16	27.6	58.2	1293.2	586.8	1189.6
CEA096	675.4	213.6	897	21.6	10	27.4	1110.8	540.2	1155.2
CEA097									
CEA100	44.2	23.8	18.2	10.4	18.4	15.8	1599.4	1399.6	1506
CEA101	302	347.4	601.4	43.2	31.8	92.6	824.8	773	834.8
CEA102	348.2	273.2	189	31.6	45.4	25.2	835.6	823	699.8
CEA105									
CEA106	33.8	40.6	24.2	7.6	4.6	6	1006	833.6	1288.6
CEA107	54	916	66.8	10	37.2	13.6	952	789.8	986.6
CEA108									
CEA109									
CEA110	126.4	77.2	70.2	15.2	12.8	12.2	1253.8	995.6	1315
CEA111	40.6	24.2	115	4.6	6	17.4	849.4	1333.6	827.4
CEA112									
CEA113									
CEA114	916	66.8	453.2	37.2	13.6	28	652.6	423.6	476.4
CEA115									
CEA116									
CEA117									

Study No	GMCSF pre	GMCSF post	GMCSF 6weeks	PlGF pre	PlGF post	PlGF 6weeks	SDF pre	SDF post	SDF 6weeks
CEA119	77.2	70.2	50.2	12.8	12.2	22.2	560	746.6	776.2
CEA120	419.6	22.4	9	23	9.2	11.8	1354.8	791.8	1164.2
CEA126									
CEA127									
CEA128	145.6	92.8	61.8	18.2	6.6	16.8	535.4	712	766.8
CEA129									
CEA130	16.4	94.8	8.2	10.2	17	9.4	1068.2	782	747
CEA131	175.6	204.8	2753.8	143	120.6	343	1214.8	1104.4	1140.2
CEA132	265.2	122.8	415.8	22.6	21.2	30.8	883.2	645	854.8
CEA133									
CEA134	5878	714.4	916.6	426.6	61.8	71	799.6	661.6	717.6
CEA135									
CEA136									
CEA137									
CEA138	115	76.2	27.2	17.4	11.2	15.2	911.4	642.2	906.4
CEA139	453.2	453.6	412	28	34.8	23.4	848.2	630.6	780.8
CEA140	50.2	90	24.2	22.2	24.4	7.4	1005.2	1103.6	1000.8
CEA141	76.2	27.2	54	11.2	15.2	10	461.6	246.2	477
CEA142	453.2	412	126.4	34.8	23.4	15.2	777.8	445.6	772.6
CEA143	90	24.2	187.6	24.4	7.4	25.6	488.8	667.6	1086.2
CEA144									
CEA145									
CEA146									
CEA147									
CEA148									
CEA149									
CEA150									

Study No	VEGFpre	VEGFpost	VEGF 6 weeks	Restenosis 3m	Restenosis 6m	Restenosis 12m
CEA066					50	60
CEA067				49	50	50
CEA068				16		49
CEA077				16		20
CEA078				15		
CEA079				16	50	40
CEA080					40	30
CEA082				40	40	30
CEA085					16	
CEA086					20	
CEA087				16	20	
CEA088				15	16	16
CEA091				20		
CEA092				16	55	49
CEA093					20	
CEA094	12.6	4.2	10.2	20	40	70
CEA095	9.2	8.6	44.6	15	20	20
CEA096	15	10.8	19	40	50	50
CEA097						
CEA100	2.2	10.8	16.8	16	40	40
CEA101	89.8	125.6	280.8	16		16
CEA102	20	129.4	98.6	15	16	30
CEA105					16	20
CEA106	58.6	38.6	385.8	50	70	79
CEA107	26	80.2	40.8	20	20	20
CEA108				20		
CEA109						20
CEA110	216.2	331.6	298.6	50	40	40
CEA111	79.8	92.2	11	49	50	50
CEA112				16		
CEA113				16	90	80
CEA114	31.8	46.6	401.6	15		
CEA115				60	75	79
CEA116				70	50	
CEA117				50		
CEA119	40.2	10.4	11		20	
CEA120	14	9.2	171.2		16	

Study No	VEGFpre	VEGFpost	VEGF 6 weeks	Restenosis 3m	Restenosis 6m	Restenosis 12m
CEA126				16	75	
CEA127				15	16	
CEA128	11.2	32	11.8	15	16	
CEA129						
CEA130	74.6	60	29.8	15		
CEA131	37.4	30.2	43.8	15	20	
CEA132	32.4	81.8	19.6	50	50	
CEA133						
CEA134	19.8	20.8	14.2	15	50	
CEA135				15	15	
CEA136				15		
CEA137						
CEA138	154.2	35.2	26.2	16	16	
CEA139	13.8	6.2	592	49		
CEA140	36	29	13.2	16	30	
CEA141	17.4	18.4	109.4	16	16	
CEA142	7	12.2	9.2	16		
CEA143	4.2	20.4	19.4	20	30	
CEA144				15	50	
CEA145				16	16	
CEA146				16	20	
CEA147				16	55	
CEA148				15	16	
CEA149				16		
CEA150				16		

Study No	Complications	Notes
CEA066		DNA 6w
CEA067		DNA 6w
CEA068		Refused 6w
CEA077		
CEA078		Refused 6w
CEA079		
CEA080		
CEA082		Refused 6w
CEA085		
CEA086		
CEA087		
CEA088		
CEA091		
CEA092		Refused 6w
CEA093		
CEA094		
CEA095		
CEA096		
CEA097		Died Ruptured AAA(DNA)
CEA100		
CEA101		
CEA102		
CEA105	Hoarsness/Swallowing Diff	DNA 6w
CEA106		
CEA107	Bleed post-op/ Dysphasia post-	
CEA108		Refused 6w
CEA109		Refused 6w
CEA110		
CEA111		
CEA112		DNA 6w
CEA113		DNA 6w
CEA114		
CEA115		Refused 6w
CEA116		Refused 6w
CEA117		DNA 6w
CEA119		
CEA120		
CEA126		DNA 6w

Study No	Complications	Notes
CEA127		
CEA128		
CEA129		PreCABG, refused 6w
CEA130		PreCABG
CEA131		
CEA132		
CEA133		DNA 6w
CEA134		
CEA135		Refused 6w
CEA136		DNA 6w
CEA137		Refused 6w
CEA138		Axil/Sub stent
CEA139		L CEA 06
CEA140		
CEA141		PreCABG
CEA142		
CEA143	Post op CVA	CVA 03 R sided,MI 01
CEA144		DNA 6w
CEA145	Post-op TIA, dif swollowing	Aorto-bifem, DNA 6w
CEA146		Refused post and 6 weeks
CEA147		CABG 2004, AF Aug 05, DNA 6w
CEA148		CABG 1994, refused 6w
CEA149		Pre AVR, refused 6w
CEA150		CABG 06, refused 6 w